

ECUT

ENERGY CONVERSION AND UTILIZATION TECHNOLOGIES PROGRAM

Biocatalysis Project Annual Report FY 1988

March 1989

Sponsored by

Energy Conversion and Utilization Technologies Division
Office of Energy Systems Research
U.S. Department of Energy

Through an agreement with

National Aeronautics and
Space Administration

by

Jet Propulsion Laboratory
California Institute of Technology
Pasadena, California

The Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology, for the United States Department of Energy through an agreement with the National Aeronautics and Space Administration (NASA Task RE-152, Amendment 307; DOE Interagency Agreement DE-A101-86CE90239).

The Biocatalysis Project focuses on resolving the major technical barriers that impede the potential use of biologically facilitated continuous chemical production processes.

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights.

Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

ABSTRACT

The Annual Report presents the fiscal year (FY) 1988 research activities and accomplishments, for the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Division. The ECUT Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology. The Biocatalysis Project is a mission-oriented, applied research and exploratory development activity directed toward resolution of the major generic technical barriers that impede the development of biologically catalyzed commercial chemical production. The approach toward achieving project objectives involves an integrated participation of Universities, Industrial Companies and Government Research Laboratories.

The Project's technical activities were organized into three work elements:

The Molecular Modeling and Applied Genetics work element includes research on modeling of biological systems; developing rigorous methods for the prediction of three-dimensional (tertiary) protein structure from the amino acid sequence (primary structure) for designing new biocatalysis; defining kinetic models of biocatalyst reactivity; and developing genetically engineered solutions to the generic technical barriers that preclude widespread application of biocatalysis.

The Bioprocess Engineering work element supports efforts in novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yields and lower separation energetics. Results of work within this work element will be used to establish the technical feasibility of critical bioprocess monitoring and control subsystems.

The Bioprocess Design and Assessment work element attempts to develop procedures (via user-friendly computer software) for assessing the energy-economics of biocatalyzed chemical production processes, and initiation of technology transfer for advanced bioprocesses.

FOREWORD

For further information about the ECUT Biocatalysis Project, contact:

Dr. Leonard Keay,
Biocatalysis Program Manager
Energy Conversion and Utilization
Technologies (ECUT) Program
Conservation and Renewable Energy
Department of Energy CE-12
Forrestal Bldg. 5E-052
1000 Independence Avenue, S.W.
Washington, D.C. 20585
(FTS) 896-1508 (FTS) 896-1507

Dr. Minoo N. Dastoor,
ECUT Biocatalysis Project
Project Manager
Jet Propulsion Laboratory
California Institute of
Technology
M/S 125-112
4800 Oak Grove Drive
Pasadena, CA 91109
(818) 354-7429
(FTS) 792-7429

CONTENTS

I.	EXECUTIVE SUMMARY	1
II.	PROJECT DESCRIPTION	3
	A. THE ECUT MISSION	3
	B. HISTORICAL BACKGROUND	3
	C. RELEVANCE	6
	D. GOAL AND OBJECTIVE	7
	E. PROJECT STRUCTURE AND ORGANIZATION	8
	F. MANAGEMENT SUPPORT FUNCTION	9
	G. TASK DESCRIPTIONS	10
III.	FY88 TASK ACCOMPLISHMENTS	23
	A. Molecular Modeling and Applied Genetics	23
	B. Bioprocess Engineering	36
	C. Process Design and Analysis	54
IV.	PUBLICATIONS	61
V.	GUIDELINES FOR PROPOSALS	71

FIGURES

2-1.	ECUT Role in Energy Conservation Research and Development	4
2-2.	ECUT Program: Projects and Lead Center.	5
5-1.	Proposal Evaluation Process	74

SECTION I

EXECUTIVE SUMMARY

The Biocatalysis Project sponsored by the Energy Conversion and Utilization Technologies (ECUT) Division of the U.S. Department of Energy (DOE) is an applied research effort focused on providing the enabling technology base for new bioprocess applications which have substantial energy implications. The primary mission of DOE-ECUT is to provide, for the nation, a bridge between basic research and industrial application for several technologies (e.g., high temperature materials, tribiology, combustion and biocatalysis) which have the potential for making positive energy contributions. Furthermore, the ECUT mission hopes to strengthen the future U.S. competitive position by conducting research directed towards exploiting appropriate advances in basic research in which the U.S. has demonstrated leadership.

As the national lead center, the Jet Propulsion Laboratory of the California Institute of Technology serves as the technical manager for the Biocatalysis Project. The primary objective of the Project is to resolve the critical technical constraints (e.g., poor productivity, high separation energetics, presence of aqueous medium, lack of design tools) that impede the utilization of biocatalysis for the production of chemicals and materials. To achieve this objective, the Project supports generic applied research as well as development of predictive theoretical models (that are experimentally verifiable) which address the technological barriers to the commercial utilization of biochemical catalysis.

The ECUT Biocatalysis Project consists of three major work elements, each addressing a key technical component of the required enabling technology base. The selection as well as the relationship between each of these three work elements has been defined by their scale of action. The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysis and pursuing practical applications of basic molecular biology research findings. Computer graphic models have been advanced which predict the dynamic behavior and intramolecular conformational changes of enzymes (biocatalysts), including effects of temperature and biocatalytic inhibitions. Advances in Applied Genetics have focused on the issue of biological information (plasmid) stability. The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between cellular scale events and macro-level parameters. Laboratory tests using advanced fluidized bed bioreactors, novel multi-membrane bioreactors and chemical transformation in organic solvents has substantially increased rates and extent of chemical synthesis by solving the chronic and ubiquitous problem of product feedback

inhibition. The Process Design and Analysis work element focuses on developing user friendly computer programs which assess the energetics and economics of biocatalytic chemical production processes. Modular computer programs have been developed which provide capabilities for rapid assessment of energy expenditures and costs for unit operations within biocatalytic processes.

SECTION II

PROJECT DESCRIPTION

A. THE ECUT MISSION

The Energy Conversion and Utilization Technologies (ECUT) Program seeks to:

- (1) Monitor advances in basic scientific research and evaluate emerging technologies for applicability to energy conservation.
- (2) Conduct exploratory development and establish feasibility of innovative, or revolutionary, conservation concepts.
- (3) Effect technology transfer to DOE end-use conservation programs and/or to private industry.
- (4) Identify energy conservation research needs and develop programmatic direction in basic research.

In fulfilling these aims ECUT acts to transmit power of basic research to the wheels of industry (Figure 2-1). There are many ways to achieve these aims and since its establishment ECUT has chosen to support long-term, high-risk generic research and exploratory development for generation of more energy-efficient materials and processes. The Biocatalysis Project is one of five projects that make up the ECUT Program (Figure 2-2).

B. HISTORICAL BACKGROUND

The Biocatalysis Project was established in 1980 as the Chemical Processes Project with the Jet Propulsion Laboratory as the lead center. At that time the Project had two work elements: (1) Catalysis (chemical catalysis and biocatalysis) and (2) Separation. Within these work elements several small contracts were completed that evaluated the energy efficiencies and potential applications of relatively new separation processes, such as supercritical extraction, membrane separation, and chromatography. Research was also started at the California Institute of Technology (Caltech) on chemical catalytic behavior models and kinetics of expression in recombinant-DNA microorganisms.

In 1982, the ECUT Program was reorganized and the Chemical Processes Project was renamed as the Biocatalysis Project. As the name suggests, the Project emphasis was also changed to stress biocatalysis as the primary research focus. Its work elements were modified to include: (1) Molecular Modeling and

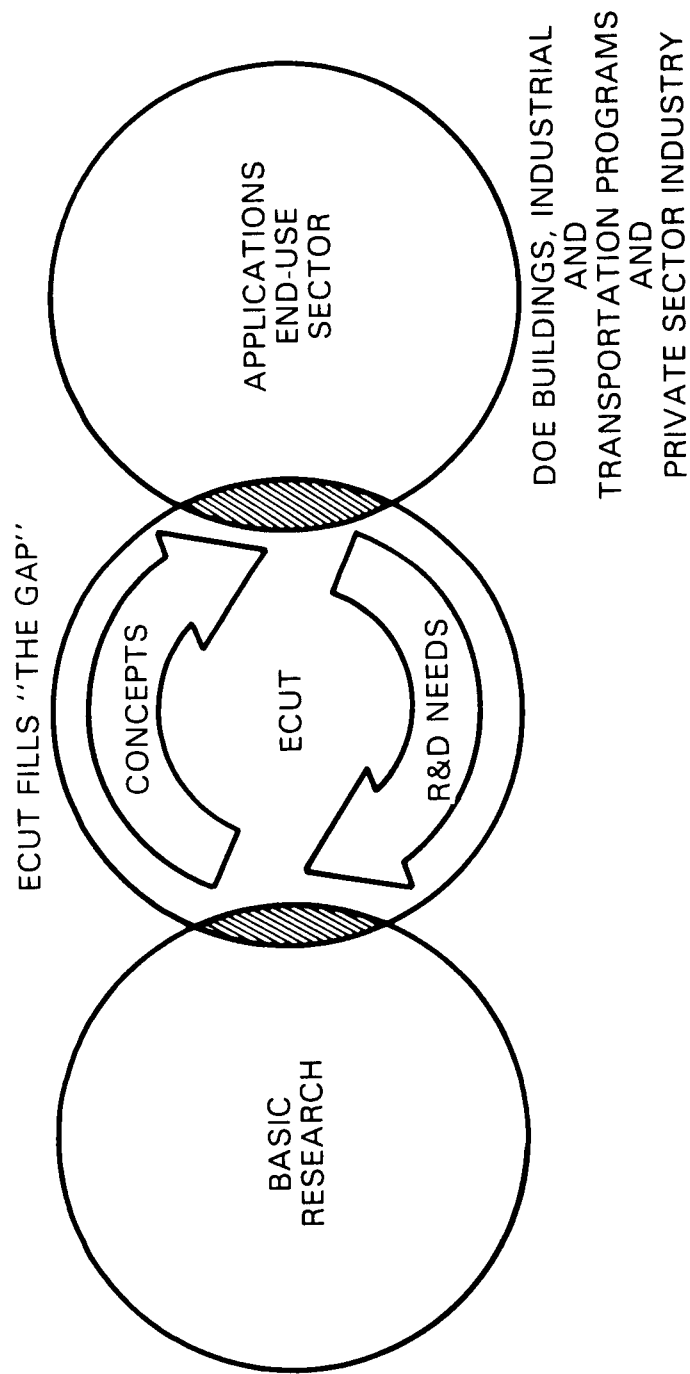


Figure 2-1. ECUT Role in Energy Conservation Research and Development

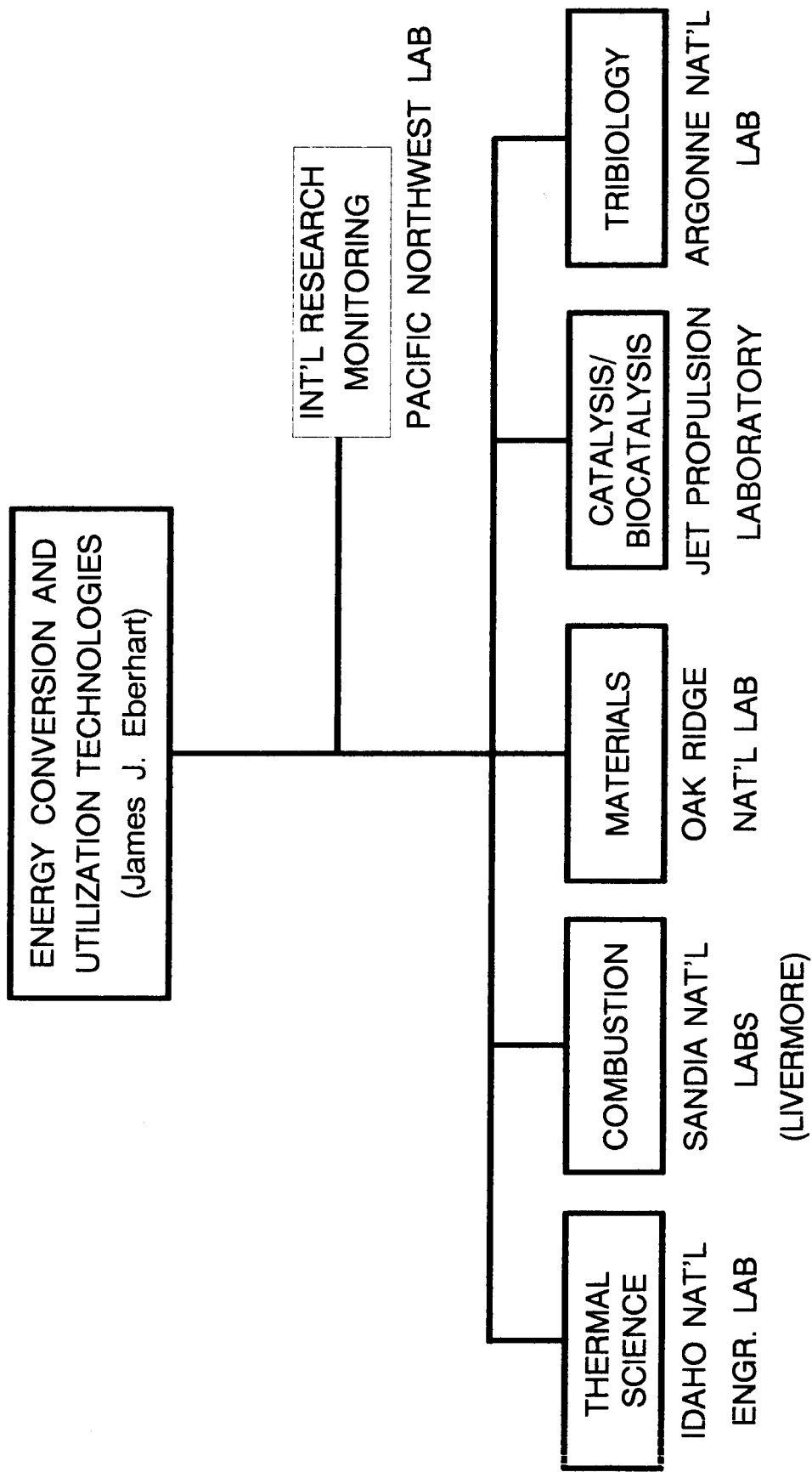


Figure 2-2. ECUT Program: Projects and Lead Center

Applied Genetics, (2) Bioprocess Engineering, and (3) Process Design and Analysis. Each of these elements addresses a key technical component necessary for the development of more advanced and efficient bioprocesses. These three work elements detail a systematic progression of understanding starting with the basic understanding of biocatalytic mechanisms at the molecular level, to micro-level effects on process parameters in reactors (including effects of integrated separation processes), and finally to the development and assessment of new process concepts for technology transfer to the industrial sector. With this information the production of large-volume, low-cost, energy-intensive chemicals from renewable resources may be a practical alternative in the future.

The selection as well as the relationships between each of the work elements has been defined by their scale of action. Research activities are conducted at various scales of action or specific size dimensions. Hence Work Element (1), Molecular Modeling and Applied Genetics, includes research activities at a molecular and cellular level, i.e., a scale of 1 μm and smaller. To successfully exploit the findings at the molecular and cellular level towards scale-up, Bioprocess Engineering, Work Element (2), includes research in the area of engineering kinetics and control as well as novel concepts in reactor design. The scale in this work element is generally at the 1 meter dimension, or at the reactor level. Finally, Work Element (3), Process Design and Analysis, focuses on activities that operate at the entire process level (0.4 hectares or more). A central tenet subscribed to by the ECUT Biocatalysis Project is that in the acquisition of an appropriate enabling technology, it is mandatory to integrate crucial research findings within the various work elements and transfer the findings to the private sector.

C. RELEVANCE

Three main conclusions can be stated regarding the competitive status and future potential of the United States Petrochemical Industry:

- (1) Many United States industries are losing their competitive edge as the global economy is expanding; the U.S. chemical processing industry is just such an example. Since 1984, the United States share of world chemical exports has decreased from 16.8 to 13.5%.
- (2) The United States must take advantage of its competitive strengths if it is to compete effectively in the future. This includes the application of its world renowned capabilities in basic science to commercial applications. Biotechnology is an example of scientific research whose application has tremendous

potential for commercial application.

- (3) Although biotechnology is being applied towards the production of specialty chemicals by the chemical industry, its application to the far larger commodity chemical market is currently considered too long term and risky to attract research and development dollars. This is sensible from the perspective of each individual firm, but it is unclear whether the specialty chemical markets can sustain all of the competitors who are restructuring their operations towards specialty chemical production. A key government role in the future competitiveness of the industry should be the support of generic research which can be applied to the development of economical commercial processes.

The application of biocatalytic processes to the production of large scale chemicals would capitalize on the three major areas in which the United States currently exerts leadership and whose successful exploitation would contribute to its global competitive position in the future. First, the United States is considered a world leader in basic research advances and our lead in molecular biology and biochemistry is unquestioned. Commercial utilization of that lead could increase the competitive advantage of our domestic chemical industries. Second, we can exert our leadership further by using the tools of macromolecular modeling and energy optimization to design entirely novel biocatalysts and to test such catalysts through computer simulation. Such a design strategy would be useless without a means for inexpensive synthesis; however, the newly developed techniques of site-directed mutagenesis provide the methodology to synthesize and test experimentally these design principles. The use of this design strategy would enhance our productivity in the future by significantly and intelligently reducing the time spent on experimental and empirical procedures in the laboratory. Third, the United States is one of the most efficient and successful producers of agricultural products in the world. Because biomass is often the feedstock for the biocatalyst production of organic chemicals, we have a clear competitive advantage in biomass production within our domestic agricultural sector.

D. GOAL AND OBJECTIVE

- (1) Goal. The goal of the ECUT/Biocatalysis Project is to exploit the United States' competitive advantage in biotechnology by facilitating the production of chemicals efficiently via biocatalytic processes. The project supports high risk applied generic research and development aimed at advancing an enabling technology base that will allow the rational development and

scale-up of large biocatalyst chemical production processes. The Biocatalysis Project does this by conducting exploratory development on, and by establishing the feasibility of, novel, innovative or revolutionary basic research advances in biotechnology.

- (2) Objective. To meet this goal, the Biocatalysis Project's approach is to focus on developing predictive models and supporting novel bioprocessing concepts which can be utilized by commercial producers for large scale chemical production. In addition, the strategy of the Biocatalysis Project is to reduce production costs by increasing product yields, by increasing reactor productivity, and by decreasing energy requirements for production. These concerns are generic in a very large number of biocatalytic processes.

E. PROJECT STRUCTURE AND ORGANIZATION

The activities of the Biocatalysis Project have been incorporated as three major work elements and a Management Support Function. Each work element addresses a key technical component for the development of the enabling technology base to produce large volumes of low priced, energy intensive bulk chemicals. The three major work elements and their supporting tasks are:

- (1) The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings. The primary roles of this work element are: to model biological systems and develop rigorous methods for the prediction of three dimensional structure from the amino acid sequence of proteins for design of new biocatalysts, to provide a data base for defining kinetic models of biocatalyst reactivity, and to develop genetically engineered solutions to the generic technical barriers that preclude widespread applications of biocatalysis. FY 1988 tasks included:
 - (a) Biocatalysis by Design.
 - (b) Metabolic Engineering.
 - (c) Protein Engineering for Nonaqueous Solvents.
 - (d) Chromosomal Amplification/Gene Fusion.
 - (e) Hyperproduction and Secretion of Polyphenol Oxidase.

(f) Biological Separation of Phosphate from Ore.

- (2) The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between molecular scale events and macro-level parameters. These parameters are required for designing scaled-up biocatalyzed chemical production processes and involve efforts in the design of novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yield and lower separation energetics. Additionally, results of work within this work element will be used to establish the technical feasibility of critical bioprocess monitoring and control subsystems. FY 1988 tasks included:

- (a) Immobilized Cell System for Continuous, Efficient Biocatalyzed Processes.
- (b) Multimembrane Bioreactor for Chemical Production.
- (c) Biocatalyzed Hydroxylation in Organic Solvents.
- (d) Integrated Biological-Chemical Process for Production of 2,3 Butanediol.
- (e) Separation by Reversible Chemical Association.
- (f) Enzyme Catalysis in Non-aqueous Solvents.
- (g) Multiphase Fluidized Bed Bioreactor.

- (3) The Process Design and Analysis work element provides overall assessments, via energetic and economic analysis of biocatalyst chemical production processes, and initiation of technology transfer for advanced bioprocesses. FY 1988 tasks included:

- (a) Bioprocess Synthesis, Integration, and Analysis.
- (b) Assessment: Biotechnology and Chemical Production.

F. MANAGEMENT SUPPORT FUNCTION

The Management Support function has three areas of responsibility: Task Management and Planning; The Guidance and Evaluation Panel; and Industry Technology Transfer.

- (1) The Task Management and Planning area administers and coordinates the task elements of the Project. These responsibilities include: developing statements of work and the evaluation criteria for

Project tasks; defining the sequence of accomplishments that will achieve Project objectives; monitoring and evaluating in-house and contract research progress; preparation and implementation of Annual Operating Plan (AOP), technical multi-year plans and budgets; personnel assignments; identifying new areas of research and issues to be studied; timely publication of Project research results and developments and their dissemination to DOE headquarters, industry and other researchers.

- (2) The Guidance and Evaluation Panel includes industrial and academic representatives who are leading authorities in the science and technology of biocatalysis. The panel is responsible for reviewing ongoing activities and future project plans as well as evaluating the Project's performance.
- (3) The Industry Technology Transfer task is to ensure strong, interactive relationships between the Biocatalysis Project and industry that will assist the transfer of the enabling technology required by the private sector for the production of chemicals via biocatalysis.

G. TASK DESCRIPTIONS

(1) Molecular Modeling and Applied Genetics

The Molecular Modeling and Applied Genetics work element is comprised of six research tasks: Biocatalysis by Design, Metabolic Engineering, Protein Engineering for Nonaqueous Solvents, Chromosomal Amplification/Gene Fusion, Hyperproduction and Secretion of Polyphenol Oxidase, and Biological Separation of Phosphate from Ore. The focus is on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings.

- (a) Biocatalysis by Design (W.A. Goddard, III, California Institute of Technology). A critical problem in the design of new biological systems (biocatalysts and biopolymers) is the limited current capability for predicting the structure and properties of the new system. It is necessary to be able to predict secondary and tertiary structure on the basis of amino acid sequences, or primary structure (which can be experimentally determined) because physical and chemical properties depend on the total protein structure. Then, since the biocatalytic function or other

critical property of certain proteins and the corresponding total structure would be known, a new biological system could be designed and synthesized to perform only those specific functions that are desired on the basis of structure and functional characteristics.

Prediction of protein structure is being investigated by three approaches that combine random fluctuations with molecular dynamics to allow molecular forces to guide optimization of local structure. (i) Full optimization is where a combination of stochastic (Monte Carlo) and deterministic molecular dynamics methods are used to search for energy minima of proteins. Although only the amino acid sequence is needed in this approach, several years of research and development are expected. (ii) Conformationally constrained optimization is where low-resolution x-ray data are used to provide constraints for Monte Carlo and molecular dynamics to reduce the time to determine structure. (iii) NMR pairwise-constrained optimization is where pairwise distances from two-dimensional NMR results are used in conjunction with molecular dynamics and energy minimization to predict optimum protein structures that are consistent with the experimental information.

A general approach to biocatalysis will be developed, using the tools of macromolecular modeling with energy optimization to design completely new biocatalysts and to test the design concepts through computer simulation. In order to allow straightforward experimental synthesis and testing of the theoretical designs, systems whose gene sequences can be obtained from site-directed mutagenesis of available clones (or else from machine synthesis) will be utilized.

A model for this study is the design of catalysts for selective oxidation of particular organic substrates. Enzymes for which there are abundant experimental data on structure and kinetics (e.g. such as cytochrome P-450, a selective oxidase) will be investigated. As the effort progresses to the point where designs are confirmed with both simulation and experimental studies, entirely new systems that may be small enough to be synthesized in other ways will be studied.

Major goals of this study will involve the following. (i) Designing modified pockets in available enzymes for selective binding of simple chemical substrates (alkenes, alkanes, aromatics, alcohols, etc.). The selectivity of the new bonding pocket can be tested both by simulation and experiment. (ii) Developing strategies for covalent attachment of modified enzymes in particular configurations on appropriate supports. The value of these strategies can be tested with experiments. (iii) Developing approaches for eliminating the bulk of the protein to achieve a stable active site complex having the same (or better) activity. This complex would be covalently attached to a support. (iv) Designing a semiconductor support that with proper doping could serve to reduce (or oxidize) the active site at appropriate steps in the reaction sequence. Control would then be provided by appropriately adjusting the external potential to the support. (v) Designing a catalyst monitor system using a related, modified enzyme with a pocket designed to recognize product (or particular intermediate). This enzyme would be covalently attached to the gate for a Field Effect Transistor (FET), so that a sensitive monitor of catalyst operation can be developed.

- (b) Metabolic Engineering (J.E. Bailey, California Institute of Technology). Metabolic engineering refers to the directed rearrangement or augmentation of metabolic pathways, using recombinant DNA techniques, in order to provide overproduction of metabolites or metabolic derivatives. Changing the enzyme makeup of the cell by genetic engineering will have secondary effects due to the cell's response to unnatural metabolic balance, often mediated through shifts in pathway branching and through ATP and reduced pyridine nucleotide levels. Experimental investigations of two different recombinant microbial systems with altered uptake and metabolic systems will be initiated. In particular, the growth and phosphorous metabolism properties of Saccharomyces cerevisiae with enhanced glucose phosphorylating activity provided by plasmid-encoded proteins will be studied. Alteration in metabolism of Escherichia coli with amplified dihydrofolate reductase (DHFR) activity, again provided by expression of a plasmid gene, will be investigated.

The objectives of these experiments are to investigate the extent to which cellular metabolism is sensitive to amplification of individual activities in certain key positions in metabolism, to develop and demonstrate powerful experimental methods for investigation of metabolic perturbations (primarily ^{31}P in vivo NMR), and to develop data for use in mathematical modeling studies of metabolic engineering. Several different mathematical approaches to analysis of cell metabolism will be pursued as part of this research. A detailed single-cell kinetic model of recombinant Escherichia coli will be enhanced to include more details of particular metabolic pathways, especially as pertaining to DHFR activity. In addition, global sensitivity analysis methods will be applied to explore those parameters in this model which are most important in simulated cell metabolism and growth kinetics. In addition, research will continue on mathematical frameworks for estimating a priori overall cell response to metabolic engineering and for analyzing experimental data on altered metabolic pathway flows in a systematic, powerful fashion.

- (c) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, California Institute of Technology). The industrial applications of biocatalysts have been severely limited by constraints on the solvent environment of proteins, which normally require an aqueous medium for effective operation. With the advent of convenient methods for altering the amino acid composition and for synthesis of entirely new proteins, it is worthwhile to consider engineering proteins that would be effective in nonaqueous solvents. However, the success of a rational design procedure for constructing proteins to use in organic solvents depends on understanding relationships among various factors: amino acid sequence, secondary and tertiary protein structure, and activity and stability in nonaqueous solvents.

The goal of this research is to begin to define these relationships, by implementing an integral and iterative protein engineering approach based on the model hydrophobic protein crambin. The unusual stability and solubility of crambin in a wide range of nonaqueous solvents will be investigated. The structures, stabilities and solubilities of crambin mutants will be measured

and correlated with specific alterations in the protein amino acid sequence. This research includes studies of the properties of crambin, design of new mutant crambins by computer/molecular graphics, production of new mutants by site-directed mutagenesis, and detailed NMR studies of crambin stability and response to nonaqueous solvents. The results of this work will be used to formulate a set of criteria and methods for rational design of proteins to be used in the presence of nonaqueous solvents.

- (d) Chromosomal Amplification/Gene Fusion (G. Bertani, Jet Propulsion Laboratory). Although genetic engineering techniques can be used to increase micro-organism productivity, e.g., to produce larger amounts of a chemical in a fermentation process, the genetic information (that results in higher production) may be lost as the microorganism multiplies. The objective of this activity is to determine if the desired genetically-engineered component (plasmid or associated gene) can be inserted into the chromosome and amplified in place. Since the desired genetic information (recombinant trait) would then be in the microorganism chromosome, it could not be easily lost as the microorganism multiplies. Thus the problem of plasmid stability could be addressed. Direct physical (as opposed to genetic) evidence for the insertion of the plasmid into the bacterial chromosome has been obtained.

The stability of bacterial strains resulting from this work will be compared with the stability of strains created by conventional plasmid splicing techniques. While laboratory strains of Escherichia coli are being used in this research, the general principles involved could easily be applied to other types of bacteria.

- (e) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). The objectives of this research are (i) to purify intracellular and extracellular polyphenol oxidases from Coriolus versicolor maintained in liquid culture and (ii) to determine if intracellular polyphenol oxidase is either de novo synthesized or activated. The approach is to (i) establish the time courses for the appearance of intracellular and extracellular oxidases and to subsequently determine specific activities, and

(ii) to establish optimum growth conditions of the microorganism for maximum production of polyphenol oxidase, including determination of cofactor requirements.

- (f) Biological Separation of Phosphate from Ore (R.D. Rogers, Idaho National Engineering Laboratory). The phosphate industry utilizes about 0.3 quads/yr for the separation of phosphate from apatite ore. Therefore this task is directed toward development of bioprocessing for solubilization and separation of phosphate from ore. The specific objectives of the proposed research are to: (i) define a microbiological system which will extract phosphate from its ore; (ii) develop a basic understanding of the biochemical mechanisms involved; and (iii) through the use of modern biotechnology develop a bioprocessing system for transfer to the phosphate industry. If a more efficient recovery process can be developed, it could also be applied to phosphate mine waste because ore containing <26% phosphorus is currently considered waste and is used as mine backfill.

The approach will be to gain a thorough understanding of the biochemical interactions which cause the microbial release of phosphate from its ore. This will be accomplished by screening microorganisms obtained from areas of high phosphate content (i.e., phosphate mines, process waste streams, fertilized agricultural lands, etc.) for their ability to solubilize phosphate. Those organisms which are positive for the desired trait will be selected for further biochemical studies. When a better understanding of the mechanism of solubilization has been established, the work will be focused on development of methods to enhance production. Enhancement could be through physical/chemical stimulation, genetic manipulations or both. The next steps will be the gradual scale-up of the process. During each of these steps, evaluations of the most efficient engineering methodology will be made.

(2) **Bioprocess Engineering**

This work element consists of seven tasks: Immobilized Cell System for Continuous Efficient Biocatalyst Processes, Multimembrane Bioreactor for Chemical Production, Biocatalyst Hydroxylation in Organic

Solvents, Integrated Biological-Chemical Process for Production of 2,3 Butanediol, Separation by Reversible Chemical Association, Enzyme Catalysis in Nonaqueous Solvents, and Multiphase Fluidized Bed Bioreactor. They are primarily concerned with the definition of basic engineering relationships between molecular and micro-scale events and macro-level parameters required to design and scale up bioprocesses.

- (a) Immobilized Cell System for Continuous Efficient Biocatalyst Processing (C.E. Scott, Oak Ridge National Laboratory). To decrease energy consumption and capital equipment costs, it is necessary to increase productivities of fermentation chemical processes significantly. Therefore, the goals of this task are to enhance productivity and operability of a fluidized bed reactor system containing immobilized microorganisms, and to investigate bioreactor dynamics, including the formulation and investigation of kinetic properties of biocatalyst particles to lead to a better understanding of reactor behavior and control predictability. The reactor consists of a column containing immobilized cells; the substrate (glucose) and nutrients are passed into the bottom of the column and the product ethanol (in water) is continuously withdrawn from the top. The primary advantage of this type of bioreactor is increased rates of fermentation resulting from the high concentration of cells in the reactor.
- (b) Multimembrane Bioreactor for Chemicals Production (M.L. Shuler, Cornell University). Immobilized cell reactors offer the potential to greatly improve volumetric productivities for bioreactors. An important form of such reactors is that of entrapping cells between membranes. Scale-up of such units can lead to problems with the transfer of sparingly soluble gases (e.g., O_2 and CO_2). These gas transfer problems can result in a loss of productivity due to starvation for gaseous nutrients or in the physical disruption of the membrane reactor due to pressure increases caused by incomplete removal of by-product gas (e.g., CO_2). Although membrane reactors are satisfactory for the retention of cells, the true advantages of membranes are incompletely utilized in current configurations. The use of membrane selectivity for in situ product recovery offers the potential to simplify process design and increase reactor productivity by avoiding often encountered

problems with product inhibition.

A multimembrane reactor concept is suggested. Four layers exist: one for cell entrapment, one for substrate flow, one for gas flow, and one for the flow of an extractant which selectively removes product. Task efforts include (i) development of a mathematical model relating the micro environment to reactor performance, (ii) development of a process simulator from the mathematical model, (iii) experiments to validate the model and to monitor the interrelationship of cell physiology to immobilization, and (iv) evaluation of long-term reactor operation.

- (c) Biocatalyzed Hydroxylation in Organic Solvents (A. Klibanov, Massachusetts Institute of Technology). As discussed earlier, one of the major obstacles in utilization of enzymes as practical catalysts in organic chemistry is the necessity to carry out enzymatic reactions in water which is a poor medium for most industrial chemical processes. For example, the enzyme polyphenol oxidase in principle has a great potential for selective hydroxylation of aromatic compounds, an often desirable but very difficult problem in organic chemistry. However, polyphenol oxidase-catalyzed oxidations cannot be conducted in water on a preparative scale because: (i) most phenolic substrates are only slightly soluble in water, (ii) quinones, formed as the product of the enzymatic oxidation, spontaneously polymerize in water, and (iii) during that polymerization the enzyme is chemically modified and consequently inactivated.

It has recently been discovered that several different enzymes, in particular mushroom polyphenol oxidase, can efficiently function in nearly anhydrous organic solvents. Furthermore, under such conditions, polyphenol oxidase can quantitatively convert phenols to the corresponding o-quinones which then can be chemically reduced to catechols; hence the overall process affords preparative regioselective hydroxylation of a phenol. This effort will explore the mechanism, implications and preparative use of the above phenomenon. Such issues as the effect of water content and the nature of the organic solvent on the catalytic activity of polyphenol oxidase in organic media, and absolute activity (as compared to that in

water) and stability (thermal and operational) of the enzyme in organic solvents will be addressed. Polyphenol oxidase-catalyzed hydroxylation of a wide range of substrates will be examined in organic solvents. Following optimization of operational conditions, preparative production of catechols including those of commercial significance will be carried out.

As a result of this study a new methodology (polyphenol oxidase catalysis in organic solvents) for preparative selective oxidation of aromatic compounds will be developed. Also, an understanding of why mushroom polyphenol oxidase is capable of functioning in organic solvents may provide the capability to make other enzymes exhibit or acquire the same property. This would lead to a new way of using enzymes as catalysts for preparative chemical transformations.

- (d) An Integrated Biological-Chemical Process (Tsao, Purdue University). The objective of this task is to demonstrate the technical feasibility of an integrated Biological-Chemical process for the continuous production of two high volume/low value products: methyl ethyl ketone (MEK) and 1,3-butadiene from fermentable sugars. This conceptual bioprocess has been divided into two segments: production of 2,3-butanediol from fermentable sugars using an immobilized cell reactor containing living cells and selective adsorption of 2,3-butanediol from the aqueous fermentation broth using a solid adsorbent column.

Work will involve the analysis of production of optically active 2,3 butanediol in both stirred and air lift fermenters. Selective adsorption research involves the analysis of various materials as adsorbents.

- (e) Separation by Reversible Chemical Association (C.J. King, University of California, Berkeley). The objective of this task is to examine and evaluate the use of reversible chemical association, or complexation, with organic agents as a method for separating polar organic substances from dilute aqueous solutions, e.g., bioprocess product or waste streams. The goal is to obtain sufficient understanding of underlying chemical, equilibrium, and transport behavior to enable rational selection of separating agents, methods of regeneration, and methods of

implementation, as well as rational conceptual design and economic evaluation.

In the production of carboxylic acids, processes such as fermentation yield low concentrations of carboxylic acids in an aqueous multicomponent solution. The subsequent separation, purification, and concentration of the carboxylic acids is often difficult and energy intensive.

Data have been obtained for co-extraction of water when methyl isobutyl ketone (MiBK) is used as a diluent with Alamine 336 for extraction of succinic acid. Work is beginning on interpretation and modeling of co-extraction of water. Equilibrium data have been obtained for extraction of succinic acid by Alamine 336 with an alkane diluent. Now data for three diluents (the alkane, chloroform, and methyl isobutyl ketone) can be compared and interpreted. Work has been started on investigation of spectroscopic methods for interpretation of complexation in both carboxylic acid/amine and alcohol/phenolics extraction systems.

- (f) Enzyme Catalysis in Nonaqueous Solvents (H.W. Blanch, University of California, Berkeley). To permit the use of enzymes, in either free or immobilized form, to more broadly enter into the production of large scale chemicals, techniques for increasing the solubility and transport rate of organics at the site of enzyme action must be found. The approach proposed here is based on the use of a second organic liquid, in which the substrate or product is soluble to a much greater extent than in an aqueous system. The second organic liquid may be water miscible or immiscible. Such systems are used currently for the enzymatic transformation of steroids. A second aspect of the use of enzymes in nonaqueous environments is the potential to run many reactions "backwards." In cases where water is a reaction substrate, its high activity in aqueous solution generally results in a shift of equilibrium to favor the normal products of the reaction. In a non-aqueous system such reactions may be forced in the reverse direction, as water will be present at low concentrations. Examples of this type of reaction include esterification, peptide synthesis and dehydration reactions such as urea formation from $(\text{NH}_4)_2\text{CO}_3$.

In order to make the use of enzymes in non-aqueous environments a feasible possibility, several problems related to the nature of proteins must first be addressed. At low water concentrations, as are found with use of polar organic solvents, enzymes may undergo conformational transitions, which may lead to gross denaturation. Enzymes with disulfide bridges are less prone to such transitions, and the use of detergents, which form ion-pairs with the enzyme, may also reduce the effect of conformational changes. Thus part of the proposed research and development effort will focus on understanding these aspects of protein chemistry.

Two model systems are being examined to develop generic technology for this effort. The first of these is the oxidation of the cholesterol in a two-phase system employing an organic solvent with the enzyme immobilized in an aqueous phase within microcapsules. The second model system to be examined is the conversion of ammonium pyruvate to tryptophan using the enzyme tryptophanase in a micellar or liquid membrane system where reactant and product are transferred by an ionic carrier. The key factors being addressed are the yields of product, rates of product formation and mass transfer rates of reactant to the catalytic site. These data will then permit an assessment to be made of the potential of these processes for further development and large-scale operation.

- (g) Multiphase Fluidized Bed Bioreactor (B. Allen, Battelle Columbus). This task is directed toward the development of a novel, fluidized-bed bioreactor concept for the continuous production of low value, high volume chemicals. The concept incorporates the basic technical features required to minimize the effects of end-product inhibition, a fundamental biochemical/ microbiological limitation that adversely affects the economics of current bioprocess technologies. That is, end-product inhibition impacts biocatalyst activity which leads to (i) low productivity, high volume reactors; and (ii) low product concentration, capital/energy intensive aqueous separation systems. These two factors reduce the economic attractiveness of current bioprocesses for bulk chemicals production.

The bioreactor concept in this task combines the use of an immobilized biocatalyst with an in-situ,

non-aqueous sorbent separation to minimize end-product inhibition which maximizes productivity and effectively increases product concentration. This combination is achieved by the use of a multi-phase fluidized-bed (MPFB) bioreactor process. The biocatalyst is immobilized as relatively large or dense particles (dense bed or phase) which are fluidized in a conventional manner using the fermentation media. A solid or non-aqueous (insoluble) solvent phase (entrained phase) is then circulated through the dense bed to remove excreted product, i.e., in-situ separation. After leaving the bioreactor, the entrained phase is circulated to a separation/desorption unit to recover product and regenerate the entrained phase. The regenerated, entrained phase is then recycled back to the bioreactor where the sequence is repeated.

(3) Process Design and Analysis

The Process Design and Analysis work element includes development of design and process technology for scaling up bioprocesses to the pilot-plant level and overall assessments (i.e., systems analyses) of biocatalyst chemical production processes. This work element is comprised of two research tasks: Bioprocess Synthesis, Integration, and Analysis, and Assessment-Biotechnology and Chemical Production.

- (a) Bioprocess Synthesis, Integration, and Analysis (J.D. Ingham, Jet Propulsion Laboratory). The purpose of this task is to derive or synthesize a series of candidate bioprocesses and systematically conduct relevant energy-economic analyses and comparisons for added-value commodity chemicals to determine potential for process technology transfer and commercial development. These bioprocesses will be modified to include projected research advances (e.g., genetically engineered microorganisms, bioreactor modeling and verification, membrane development, and biocatalyst immobilization) for energy-economic comparative assessments.

Recent work has included assessments of processes for ethanol and citric acid using column bioreactors with immobilized cells as fluidized-bed biocatalysts. Also, computer programs for calculation of bioreactor kinetics and models for technoeconomic assessments are being developed. It has been found that the effect of increased

productivity is advantageous, but the relative advantages for various processes are strongly dependent on product concentration and the ratio of fermenter capital-related costs to total capital-related costs for the specific bioprocess. Therefore, as productivity is increased, product concentration should be maintained above about 70 g/l, and if the ratio of costs for, e.g., fermentation to feedstocks processing or purification is low, increases in productivity above process-specific critical levels do not provide significant economic advantages.

- (b) Assessment: Biotechnology and Chemical Production (Chem Systems Inc.). The objective of this work is to carry out technoeconomic assessments of specific bioprocesses where most of the new technology has already been developed, primarily in support of technology transfer activities.

SECTION III

FY88 TASK ACCOMPLISHMENTS

A. Molecular Modeling and Applied Genetics

- (1) Biocatalysis by Design (W.A. Goddard, III, California Institute of Technology). The objective of this study is to formulate models that are able to predict the secondary and tertiary structure of a protein on the bases of the amino acid sequence or primary structure (which can be determined experimentally). This would be of considerable help in designing new biocatalysis because the physical and chemical properties depend on the total protein structure.

A joint theoretical/experimental study probing the catalytic requirements and properties of Dihydrofolate Reductase (DHFR) has been undertaken as a model. Full kinetic investigation of two bacterial forms of the enzyme, from Escherichia coli and Lactobacillus casei produced nearly identical binding and catalytic profiles (work of Benkovic et al., Penn State collaborators) even though the overall sequence homology of the two proteins is only 27%.

Investigation of these proteins using the molecular facilities at Caltech offered some insights into this remarkable similarity. Detailed examination revealed that the homology of the substrate binding site (defined to be within 7 Å of bound MTX inhibitor) is, in fact, 50% when those site amino acids which have only backbone site interactions are also deemed homologous. A comparison of the total solvent accessible surfaces of the substrate binding pocket for the two proteins demonstrate that the Escherichia coli area is 93% that of Lactobacillus casei. Similarly, the NADPH cofactor binding site examined in this manner produced a sequence homology of 70% while the site surface area of the Lactobacillus casei protein is 92% that of Escherichia coli. This clearly illustrates that the same active site surface can be constructed by differing combinations of amino acids.

Work in this area continues with the focus being: (i) a comparison of the two bacterial DHFR proteins with the enzyme from a eukaryotic source (chicken liver) and (ii) simulations to probe the stability of hybrid proteins, i.e., the Escherichia coli system with selected mutations analogous to the Lactobacillus casei protein. It is believed that once a better understanding of these natural mutants is in hand, the

lessons learned will facilitate the design of selective synthetic mutants.

A second model being studied is based on a novel class of artificial receptors called Starburst Dendrimers. These polymers incorporate branch condensations of β -alanine monomers that double the number of polymerization sites at each generation. By generation five, the addition of new monomers is expected to lead to structures with interior cavities that can serve for host-guest complexation. It has not been possible, however, to obtain structural information related to these concepts.

Atomic-level simulations have been carried out to investigate the structure and properties of these dendrimer systems. The force fields employed have been tested on analogous biological systems. We have simulated three solvent models: aqueous (hydrophilic), methanol (the experimental solvent), and vacuum (hydrophobic). The simulations use a combination of heating/annealing (molecular dynamics/energy optimization) and Monte Carlo random forces (to heat up selective regions of the polymer).

To date, we have studied isolated host dendrimer systems up to seven generations and have begun simulations of host-guest complexes to investigate the application of starburst dendrimers as delivery agents. The design of chemically specific macromolecules will also be undertaken.

- (2) Metabolic Engineering (J. Bailey, California Institute of Technology). The objectives of this study are to investigate the extent to which cellular metabolism is sensitive to amplification of key individual metabolic activities, to develop and demonstrate experimental methods for investigation of metabolic perturbations primarily ^{31}P in vivo NMR, and to develop data for use in mathematical modeling for use in metabolic engineering. Several model systems (including genes for hemoglobin, hexokinase, and the pathway going from pyruvate to ethanol) are being employed to fulfill these objectives. As an outcome of this work a Metabolic Pathway Synthesis (MPS) program has been developed. The MPS program can be used to predict in a qualitative way the effect of adding or deleting enzymatic activities to or from the cellular environment, to classify pathways with respect to cellular objectives, and to extract information about metabolic regulation. MPS can be used for the identification of appropriate genotypes or genetic

modifications that will redirect metabolism towards amplified production of desirable bioproducts.

Research continues on the utilization of cloned bacterial hemoglobin from Vitreoscilla to enhance growth and product formation in aerobic bioprocesses. The cloned hemoglobin gene from Vitreoscilla has been sequenced and conforms exactly to the previously published amino acid sequence for this protein in its natural host. Portions of DNA flanking the hemoglobin gene have been removed to obtain a cassette which can now be readily inserted into expression vectors for other organisms and into different Escherichia coli expression vectors. A vector for examining the effect of cloned hemoglobin on high level expression of another protein has been completed. Preliminary findings with this vector demonstrate that active hemoglobin can be expressed using cloned Escherichia coli try promoter. The model protein on this vector is chloramphenicol acetyltransferase. This enzyme is under control of a lac promoter contained within the cytoplasm of the cell, and is easy to assay, but has no metabolic function in Escherichia coli. A number of deletions of the original cloned Vitreoscilla fragment have been prepared to facilitate future research in applying hemoglobin expression for growth enhancement in other cell types and for study of oxygen regulation of the Vitreoscilla promoter. An expression vector for Vitreoscilla hemoglobin in yeast has also been constructed. Efforts are underway to formulate an expression vector for hemoglobin in gram-positive bacteria.

Studies have been initiated on the effect of varying dissolved oxygen concentrations on the transcription and expression of the cloned Vitreoscilla hemoglobin gene. The objective of these experiments is to define the relationship between dissolved oxygen and expression of the hemoglobin gene with the native Vitreoscilla initiation sequence. Preliminary experiments have clearly shown that, with the original Vitreoscilla fragment, the level of hemoglobin expression is tightly regulated by the oxygen content in the culture.

Research on the influence of expression of different hexokinase activities from plasmid-encoded genes in recombinant Saccharomyces cerevisiae has been concluded. Intracellular sugar phosphate measurements based upon ^{31}P -NMR have been calibrated based upon analysis of cell extracts in vitro. The data has been employed to estimate the level of ATP in the cell which

is not complexed to magnesium. Data on metabolite levels is informative regarding large discrepancies between in vitro hexokinase activities and in vivo activities for glucose uptake and ethanol production in these cells. The results clearly indicate that metabolic regulation of enzyme activity in vivo can play a major role in influencing the outcome of a metabolic engineering effort, and these results strongly motivate further measurements of intracellular metabolite levels using NMR and other techniques.

Studies of recombinant Escherichia coli bioenergetics using NMR have confirmed that undissociated lactic and acetic acids rapidly penetrate the membrane. Transport of these components consequently plays an extremely important role in the level of the proton motive force across the bacterial cytoplasmic membrane.

The protein contents of Escherichia coli harboring different plasmids have been analyzed using two-dimensional analytical electrophoresis. The gel patterns have been digitized and analyzed with a sophisticated computer program. In addition to showing the relationship between plasmid number and cloned gene expression, these experiments also reveal important and interesting interactions between the plasmid and the host cell. In particular, several proteins present in the plasmid-free host are completely absent in plasmid-containing strains, and other host-cell proteins are expressed in plasmid-containing cells which are not synthesized at significant levels in the plasmid-free host. Further analysis of this information is necessary in order to ascertain the metabolic implications of these cellular responses to genetic engineering.

Experiments to characterize host-plasmid interactions and the effects of cloned gene induction in recombinant Saccharomyces cerevisiae have been done on two different recombinant systems. These systems are identical except for the cloned promoter employed to regulate expression of a cloned Escherichia coli lacZ gene. The promoters employed were the yeast GAL1 and GAL10-CYC1; the GAL1 promoter gives higher expression levels in shake flask cultures. Continuous culture experiments, each lasting more than 150 hours, were conducted using both of these strains at a dilution rate of 0.2 hr^{-1} . The fraction of plasmid-containing cells at steady state was 72% for the induced GAL1 promoter and 86% for the hybrid promoter, while the steady state biomass concentrations were 0.98 and 1.2 g/l for these two strains, respectively. However,

substantially higher cloned gene expression was obtained with the GAL1 promoter which more than compensated for the reduction in plasmid containing cell fraction and biomass levels. Thus, the volumetric productivity of the cloned β -galactosidase was approximately threefold higher at steady state for the GAL1 promoter system.

The influence of plasmid presence and cloned gene induction on the activities of key metabolic enzymes was also tested in Saccharomyces cerevisiae. Such data are important to obtain baseline information on genetic level responses to metabolic engineering which will guide more detailed studies of such responses in future research. In these experiments, the activities of fructose-bisphosphate aldolase, hexokinase, glucose-6-phosphate-dehydrogenase, and glutamate dehydrogenase were measured for cells grown under induced and uninduced conditions. Preliminary results from these experiments suggest that a significant decrease in glycolysis activity and a slight decrease in nitrogen assimilation occurs after cloned gene induction.

The objective of this study is to formulate mathematical models for analysis of directed metabolic changes accomplished by recombinant DNA methods. As an outcome of this work a Metabolic Pathway Synthesis (MPS) program has been developed. The MPS program can be used to predict in a qualitative way the effect of adding or deleting enzymatic activities to or from the cellular environment, to classify pathways with respect to cellular objectives, and to extract information about metabolic regulation. MPS can be used for the identification of appropriate genotypes or genetic modifications that will redirect metabolism towards amplified production of desirable bioproducts.

- (3) Protein Engineering in Nonaqueous Solvents (F.H. Arnold, California Institute of Technology). The goal of this project has been to demonstrate that enzymes can be engineered for improved stability in polar nonaqueous solvents. The ability to carry out biocatalysis in nonaqueous media greatly expands the potential scope and economic impact of biotechnology. For this reason, we are endeavoring to construct nonaqueous solvent-stable enzymes by site-directed mutagenesis of natural enzymes, based on a rational set of design criteria.

During the first phase of this project, we studied the natural plant protein, crambin, in an effort to understand the structural and chemical basis of its

unique stability in polar nonaqueous solvents. Our preliminary NMR studies indicated that crambin retains its three-dimensional structure in a wide variety of solvents, including glacial acetic acid, methanol, and dimethylformamide. As had been observed for proteins suspended in nonpolar organic solvents, crambin's stability to thermal denaturation was found to be strongly related to the hydrophobicity of the solvent.

We have barely begun to understand the special features of crambin that contribute to its remarkable stability in nonaqueous solvents. In order to obtain more detailed structural and dynamic information on crambin in various organic solvents, we are conducting high-resolution two-dimensional NMR experiments. Since crambin is soluble in a number of solvents, we can use NMR to identify the structural and dynamic changes that accompany transfer from one solvent to another. In order to do this, it was necessary to assign proton resonances in strategic positions throughout the molecule, and this has been largely completed. Nearly all the amide protons of the crambin backbone in helical and β -sheet regions have been assigned to specific amino acid residues. We have now begun to use these assignments to study the stabilities of individual hydrogen bonds as a function of changes in the solvent environment. These studies should prove to be very useful in pinpointing the regions of the protein that are susceptible to disruption by organic solvents. This information is needed in order to identify where to direct alterations in the amino acid sequence.

Crambin, which is itself insoluble in water, is homologous to several water-soluble plant toxins. A comparison of the amino acid sequences of these proteins and a study of crambin structure and composition led us to our first description of a set of "design rules" for proteins in nonaqueous solvents. By comparing crambin and its water-soluble homologs, we can identify the specific amino acid substitutions that nature has used to create a nonaqueous-solvent stable variant of the plant toxins. Some features that a protein should exhibit in order to be stable in nonaqueous media became clear from this work: a high degree of ordered secondary structure (internal hydrogen bonding), crosslinks between elements of secondary structure (salt bridges, disulfide bridges), and a surface that does not require large numbers of hydrogen bonds to water. If the internal elements are fulfilled by a natural protein, then alteration of surface properties to reduce interactions with water

may be all that is necessary to improve stability in nonaqueous solvents.

The next phase of this project, which we embarked upon this past summer, was to apply what we learned from crambin to the design and construction of nonaqueous solvent-stable variants of two natural enzymes, subtilisin and α -lytic protease. It is hoped that these mutant proteins could then be used to carry out peptide and protein syntheses in nonaqueous media. We have studied the structures and amino acid compositions of subtilisin and α -lytic protease and have proposed a set of mutations that should increase the stability of each in polar nonaqueous media.

- (4) Chromosomal Amplification/Gene Fusion (G. Bertani, Jet Propulsion Laboratory). The bulk of the molecular cloning work in genetic engineering has been based on the use of bacterial plasmids - small genetic elements carried by the host bacterium which are not bound to the bacterial chromosome. Another, less developed form of molecular cloning involves the insertion of the foreign gene or genes, directly onto the host cell chromosome. This can be done in at least two ways. (i) The foreign genetic material is associated with some DNA sequences which are long enough and similar enough to other sequences in the host cell chromosome so that the general recombination system of the host cell recombines them into the chromosome. A consequence of this process is the formation of a long repeat on the chromosome, on which general recombination can operate as well, with the result that the foreign gene can be excised and lost. (ii) A second method exploits the integration system of some bacterial viruses: these are able to insert their own DNA into the bacterial chromosome by a highly site-specific recombination event. The latter requires a specific enzyme, integrase, coded in the virus DNA, and a short DNA sequence, the attachment site, which is recognized by the integrase and present in both the virus and the bacterial DNA.

We have developed the latter method using the integration system of bacteriophage P2, in such a way that no active integrase remains in the system after integration has occurred. Since the DNA sequences required for integration are very short, they do not represent a good substrate for the general recombination system of the host cell. These structures are therefore extremely stable.

In addition, it is known that through accidents of recombination and/or replication, now and then, chromosomal DNA sequences can be duplicated to form so-called tandem repeats. These repeats can easily be excised and lost through general recombination, although a copy will always remain as part of the chromosome. Also, through general recombination, they can be amplified to give more than two copies in tandem. Using our system, we have shown that, by appropriate selection, strains carrying a dozen or more copies can be obtained. These strains however are unstable but they will always maintain one copy of the insert. Much of the work during the past year has involved investigation of this instability, and possible ways to control it.

The situation is relatively complicated because of the poorly known interactions between the two bacterial genes *recA* and *polA*. The former controls general recombination: when inactive, as in the mutant *recA13*, the frequency of general recombination is reduced several orders of magnitude. The latter, *polA*, is important in the repair of certain DNA damages; it is however a nonessential gene for the host. On the other hand, the *polA* function is required for the multiplication of the plasmid that we have been using in this work, a derivative of pBR322.

Our host strain carries the temperature sensitive mutation *ts-polA12*: the strain is nearly normal in respect to the *polA* function when grown at low temperature (20-30°C), and is defective at high temperature, 40-42°C.

This was arranged on purpose, since it allows the elimination of all free plasmid copies in the process of isolation of the integrated form, by raising the temperature at the appropriate time. Unfortunately, interactions between *recA* and *polA* make the combination of the two mutations inviable at the higher temperatures.

Several attempts to introduce the *recA* mutation into our integrated amplified strains failed, even though control experiments using the parental strain (without plasmid) were successful. We have therefore started investigating more precisely the properties of these amplified strains and the interaction between the two mutations.

There is another possible source of instability for chromosomally integrated plasmids: if the plasmid

starts replicating, this might interfere with the orderly replication of the bacterial chromosome. One might expect this effect to be stronger if the chromosomally integrated plasmid is present in several copies, if the direction of replication of the plasmid is opposite to that of the chromosome, and if the duplicated sequences are actively transcribed. There is also some evidence suggesting that the strength of these effects might depend on the position of the integrated plasmid in respect to the replication origin of the chromosome, the closer the stronger.

- (5) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). Wood-decay within forests, a renewable photosynthetic energy source, is caused primarily by Basidiomycetous fungi, e.g., white-rot fungi which possess the ability to degrade lignin, cellulose and hemicellulose, the main polymers of wood. In the case of at least one of these fungi, Coriolus versicolor, this ability results from the fungus' capacity to elaborate both extracellular cellulolytic and ligninolytic enzymes. Coriolus versicolor can mediate synthesis and secretion of these enzymes either in situ or in vitro. With regard to the latter, at least one of the enzymes, polyphenol oxidase (PPO) makes its appearance within a culture medium in a highly time-dependent fashion, i.e., extracellular enzyme begins to occur 7 days post-inoculation. Presumably, extracellular PPO originates via secretion of intracellular PPO. However, this presumption requires verification. Because PPO, an enzyme capable of converting diphenols to diquinones and oligomerizing syringic acid, appears to be inducible, it is conceivable that Coriolus versicolor could be a model for achieving over-production of enzymes. In addition, the system, which can be "scaled-up" to industrial levels of enzyme production, seems to be one in which the regulation of synthesis and/or secretion of both cellulolytic and ligninolytic enzymes can be investigated separately, i.e., experiments can be designed where results may lead to the independent control of synthesis and/or secretion.

The objectives of this project are: i) to develop reliable and rapid methods for the purification of polyphenol oxidase, ii) to determine whether extracellular polyphenol oxidase results from either de novo synthesis or activation, iii) to enhance polyphenol oxidase production by substrate induction, mutant production and/or cloning, iv) to develop methods for the batch culturing of Coriolus versicolor, v) to establish the route of polyphenol oxidase

secretion, vi) to provide the metabolic requirements for polyphenol oxidase secretion and vii) to separate polyphenol oxidase synthesis from secretion. These objectives are being examined by combined electron microscopy, biochemistry, immunochemistry and molecular genetics.

To start with we can now describe the growth kinetics of Coriolus versicolor cultured in a defined liquid medium for 16 days at 25 ± 2 C. We have also determined when in this growth cycle both the intracellular and extracellular PPO activity appears and when 280 nm absorbing substances for both non-dialyzed and dialyzed mycelial homogenates and growth medium, occur respectively. Knowledge of the intracellular time course for changes in PPO activity enabled us to administer $1.9 \mu\text{g}$ of the protein synthesis inhibitor, cycloheximide per ml of culture medium and subsequently quantify both intracellular PPO activity and 280 nm absorbing substances. When administered at day 0 and the mycelia were harvested at day 12 of culture, it was observed that the cycloheximide significantly impaired intracellular PPO activity thereby providing the opportunity to perform "labeling" experiments. These involved quantifying [^{14}C]-leucine incorporation into trichloroacetic acid-insoluble protein in the presence and absence of cycloheximide and correlating these results with the effects of cycloheximide on intracellular PPO activity. These experiments served to establish whether PPO was synthesized de novo or activated. In this connection, the successful banding of PPO in a CsCl_2 gradient was achieved as another means of differentiating between synthesis or activation by density labeling PPO with D_2O .

Concomitant with the synthesis vs. activation question, experiments were designed and performed which might result in a protocol that would purify PPO. The necessity for purification centers about the requirement to produce antibody which could be used for antigen localization in tissue sections of hyphae prepared for electron microscopy as well as for establishing PPO as a product of in vitro translation. We have now determined that dialysis of growth medium (source of extracellular PPO) followed by a $0 \rightarrow 30\%$ ammonium sulfate "cut" resulted in a 7 fold purification of extracellular PPO. Furthermore, extracellular PPO could be exchanged with the counter ion of the anion exchange resin DEAE-A50.

At the 7th International Biodeterioration Symposium we presented a description of the ultrastructure of Coriolus versicolor hyphae grown with and without the phenolic compound, catechol. This paves the way for examining the route of PPO secretion through endomembrane components.

Recent research efforts have focused upon definitely establishing whether intracellular PPO was de novo synthesized, ascertaining the substrate specificity of PPO, further purifying extracellular PPO, deriving mutants which may be over-producers of PPO, and isolating and characterizing genomic DNA as well as digesting it with restriction endonucleases in order to generate DNA fragments bearing the gene(s) for PPO. In addition, procedures were performed to ligate the genomic DNA fragment into the EcoRI site of plasmid pBR325. Lastly, we have begun to assess whether the fragment can be expressed in Escherichia coli cells harboring the recombinant plasmid.

With regard to synthesis or activation, it was observed that cycloheximide inhibited both the uptake and subsequent incorporation of [^{14}C]-leucine into TCA-insoluble protein and that this inhibition was accompanied by a significant inhibition of intracellular PPO activity. This suggested that PPO was synthesized de novo. This prompted the use of D_2O in an attempt to density label PPO coupled with CsCl_2 centrifugation of the mycelial homogenates in hopes of observing a shift in PPO density. Cesium chloride centrifugation of mycelial homogenates as a means of answering the de novo synthesis question was not successful as D_2O inhibited the activity of the enzyme.

The specific activities of PPO for three phenolic compounds, catechol, gallic acid and syringic acid have been determined for extracellular extracts. The preferred substrate for these extracts is catechol. Gallic acid and syringic acid are used in varying degrees and their preference compared to catechol is dependent on the age of the culture.

With regard to purification, a sequential protocol of dialysis \rightarrow ammonium sulfate fraction \rightarrow DEAE chromatography and gel filtration appears to be evolving for the purification of extracellular PPO. The extent of purification is being determined by following changes in PPO specific activity as well as loss of protein bands during Laemmli SDS-PAGE.

Within the past year recombinant DNA technologies have been utilized in attempting to achieve over-production of PPO. Advances in this area have lead to the following conclusions: i) Genomic DNA could be isolated and purified by CsCl_2 ethidium bromide gradients as evidenced by a distinct band upon ethidium bromide staining of agarose gel. ii) Numerous DNA fragments could be generated when genomic DNA was subjected to EcoRI digestion. iii) It appeared that an EcoRI generated fragment could be inserted into the EcoRI site of the chloramphenicol resistance gene of plasmid pBR325. iv) Agarose gel electrophoresis profiles appeared to verify the generation of a recombinant plasmid and v) Escherichia coli cells transformed with the recombinant plasmid revealed resistance to ampicillin and tetracycline, but sensitivity to chloramphenicol. Finally, assays are in progress to determine whether PPO will be produced in Escherichia coli cells. Besides these accomplishments we have isolated and purified poly-(A+) and (A-)-RNA for its use in vitro translation to obtain cell free synthesis of PPO.

- (6) Biological Separation of Phosphate from Ore (R.D. Rogers, Idaho National Engineering Laboratory). Applying biotechnology to the process of solubilizing phosphate ore has been divided into the following seven tasks: (i) selection and culturing of those microorganisms from the environment which can produce soluble phosphate from insoluble sources; (ii) determination of the biochemical phenomenon responsible for the solubilization process; (iii) calculation of process kinetics and determination of rate limiting steps; (iv) enhancement of the solubilization process of select organisms through the manipulation of external physical/chemical factors and internal genetic composition; (v) experiments to investigate the possibility of using biomechanisms for the concentration/separation of soluble phosphate from process solutions; (vi) evaluation and selection of a bioprocessing methodology which will maximize the peculiar phosphate solubilizing biochemistry of selected organisms; and (vii) collaboration with industry in the cost-shared scale-up and integration of developed methodology into their phosphate extraction process stream.

Presently, we have evaluated 860 microorganisms isolated from 54 different environmental samples, including those from phosphate mining and processing, for their ability to solubilize tricalcium phosphate (TCP) using the agar plate bioassay. This assay is

used as a screen since it only indicates if the ability to solubilize TCP is present or not. Of the isolates screened by this method, 36% of the bacteria and 19% of the fungi were able to solubilize TCP. In an initial selection, 18 microorganisms (9 bacteria and 9 fungi) were evaluated for their ability to solubilize known amounts of TCP while growing in a liquid medium. Two of these microorganisms (1 bacteria and 1 fungus) which were able to solubilize in excess of 75% of the available phosphate, were selected for studies on biosolubilization of Idaho rock phosphate ore (RP).

The studies with the RP were designed to provide data on the quantities of phosphate and calcium released, solution pH, and titratable acid, as well as the quantity and type of organic acids produced. Data from this work has shown that there is a direct correlation between the above items and the release of phosphate. The mechanism for solubilization appears to be linked to the production of organic acids. It is thought that these acids serve the two fold purpose of acidifying the solution containing the RP as well as chelating the calcium as it is being solubilized thus making it possible for solubilized phosphate to remain in solution. We have found that the fungus when grown in the presence of glucose produces gluconic acid while the bacteria produces an as yet unidentified organic acid. These findings are interesting in that gluconic acid is not a product of the tricarboxylic acid cycle but is produced by a direct enzymatic reaction with glucose.

It has been found that up to 70% of the phosphate in RP can be released at an initial loading of 100 mg RP/50 mL solution. Initial loadings of up to 500 mg have been used with increased amounts of phosphate being solubilized but at a decreased efficiency. Time course studies have shown that both microorganisms are able to commence solubilization of RP within the first 24 hr of incubation. Depending on the growth, peak solubilization has occurred anywhere from three to six days. In addition to the factor of time, there has been work designed to determine what effect soluble phosphate has on the solubilization process. When the microorganisms were grown in the presence of various concentrations of soluble phosphate it was found that at concentrations above 12 mM the solubilization of RP was decreased. This could be the result of inhibition at the molecular level or chemical change in the growth solution since an increase in pH was noted in those solutions containing higher soluble phosphate concentrations. However, the amount of phosphate

being released from RP is encouraging and certainly indicates that with further planned development the bioprocess will be useful to industry.

In addition to developing methods to solubilize phosphate, work is being conducted on methods for bioconcentration of the solubilized phosphate. The need for such a process is obvious when it is realized that while the concentration of soluble phosphate in a microbial process solution can be quite high for a biological solution, it is not sufficient for the industrial processing of phosphate. Two possible mechanisms that are currently being explored for the concentration process include the biological reduction of phosphate to either elemental phosphorus or phosphine gas and the precipitation of phosphorus from solution as dicalcium phosphate by biologically altering solution pH. Both of these mechanisms have been shown to occur under laboratory conditions.

Industry has been a direct contributor to this work both intellectually and monetarily through a technology transfer agreement. Because of this interest, scale-up of the process is being designed at the outset with an eye on industrial needs.

B. Bioprocess Engineering

- (1) Immobilized Cell System for Continuous, Efficient Biocatalyzed Processes (C.D. Scott, Oak Ridge National Laboratory). The ECUT Biocatalysis Program at ORNL seeks to increase fundamental and practical knowledge in the areas of advanced bioreactor concepts, efficient immobilized biocatalyst systems, and enzymatic catalysis in organic media. Hydrodynamics have been studied in columnar three-phase bioreactors, and the reactor concept is being extended from enhanced ethanol fermentation to continuous fermentation of neutral solvents by using a new modified biocatalyst. Fundamental measurements of diffusion in gel biocatalyst have been performed under a variety of conditions. Enhanced enzymatic activity and solubility in organic media have been demonstrated for several polymer-dye-enzyme complexes. This new approach may have general utility in solubilizing active enzymes in organics.

A fluidized-bed bioreactor (FBR) using immobilized Zymomonas mobilis to ferment dextrose to ethanol has been shown to have several strong advantages. Volumetric productivities of >60 g EtOH/L-h were observed at >99% conversion. This is a more than ten-

fold increase over conventional fed-batch reactors with yeast. In addition, a yield of 0.49 g EtOH/g dextrose (97% theoretical) was observed over a variety of flow rates, conditions and transients. The reactor was operated successfully for over 700 h without sterile feed. The dramatic improvements in these results over conventional technology are very encouraging of the promise of this system in particular and immobilized columnar reactors in general. Corporate interest has been expressed in this concept by several ethanol fermenters, one of which has provided the industrial feedstocks for the above mentioned studies.

A bioconversion of considerable interest is the anaerobic fermentation of sugars into organic solvents such as acetone and butanol by Clostridia. A difficulty of the continuous use of these microorganisms is the need for an anaerobic environment. Cell membrane fragments that will reduce oxygen to water have been developed at ORNL. These fragments can be immobilized in gel beads along with microorganisms to create an anaerobic microenvironment within the biocatalyst despite the external conditions (a patent application has been prepared for this unique new product). This will allow the development of high biocatalyst loadings for use in a three-phase FBR solvent fermentation.

Cells of Clostridia acetobutylicum have been successfully immobilized with the anaerobic membrane fragments in gel beads of carrageenan and used to produce solvents from dextrose in batch experiments. A continuous three-phase fluidized-bed reactor was tested. After 70 h, the effluent product concentration showed the development of stable solvent production instead of acidogenesis. Past the inlet, the bed was anaerobic even though an oxygenated feed was used. Glucose conversion continued within the beads and the products, listed in order of decreasing effluent concentration, were butanol, acetone, acetic acid, butyric acid, and ethanol. The use of anaerobic biocatalyst beads was successful, since the production of the desired solvents could be sustained in spite of the use of oxygenated feeds.

In order to scale-up and understand these three-phase FBRs, appropriate models are needed. Simple models using only plug-flow conditions are inadequate, but the hydrodynamics of three-phase systems are poorly understood, especially for systems producing gas, e.g., CO₂. Research in this area addresses the lack of characterization of phase holdup and backmixing for

three-phase systems in the regimes of biological interest and is expected to improve considerably the accuracy of FBR models.

A novel approach using axial electroconductivity probes was demonstrated to be a reliable method for the non-intrusive continuous measurement of the gas fraction within both non-fermenting and actively fermenting three-phase fluidized-bed bioreactor systems. In essence, both the broth and the beads are equally conductive while the gas is not. Therefore, the conductivity decreases with the amount of gas present.

Columns of 2.54-cm (ID) and 7.81-cm (ID) were fluidized with ~1 mm gel beads. There were major differences in behavior between two columns when operated at the same superficial gas and liquid velocities. In the larger column, the bubbles coalesce to an apparently stable diameter near 5 mm and the gas fraction was <3% for a range of liquid and gas flows. This indicates operation in a turbulent regime where bubble coalescence matches breakup and wall effects are relatively unimportant. In the 2.54-cm (ID) bed, the gas bubbles rapidly coalesce to span the column, causing slugging, much higher gas holdups, and increased backmixing. Differences in bed expansion were also observed between the two columns. These differences will prove important in scale-up, as most laboratory systems are small.

Conductivity was also used to estimate the mixing within three-phase FBRs from the response to a salt tracer. Particle Peclet numbers, estimated to be on the order of 10^{-2} , were found to be in moderate agreement with several literature correlations for the larger column. These parameters will be incorporated into an overall three-phase fermenting FBR model.

These results, among the first in columnar bioreactors, are corroborated by measurements made during ethanol fermentations in both columns. The hydrodynamics of the 7.62-cm column should scale to even larger systems. It is felt that larger diameter systems for high productivity bioconversions will have improved operability over the smaller fermentation FBRs that already have been successfully tested.

Biocatalyst beads can be produced by the entrapment of microorganisms or enzymes into a stabilized hydrocolloidal gel such as alginate or carrageenan. In order to determine and predict the effectiveness of such catalytic material, it will be necessary to know

the mass transport properties of substrates and products within the gel beads, especially when there are high concentrations of biocatalyst or other additives. This work clarifies some of the apparently conflicting literature reports on diffusion in gel beads.

Diffusion within the beads has been studied by measuring the transport of solutes to and from a well-stirred solution of limited volume. Tests have been performed with biocatalyst beads made from alginate and carrageenan covering a broad range of bead diameters, gel concentrations, concentration of cells and other additives, and solutes. At low gel concentration without entrapped biocatalyst, a solute of low molecular weight has a diffusion coefficient approaching that measured in water. However, with increased gel concentration, and especially with high loading of microorganisms, the diffusion coefficients are significantly decreased. An empirical relationship was developed for the diffusion coefficient as a function of microbial loading. These data can now be used in estimating mass transfer and reaction rates in gel biocatalyst.

It has been shown that some biocatalysts can be active in some organic solvents. Unfortunately, most enzymes have low solubilities in organics, and in some cases the catalytic activity is significantly affected. It therefore becomes important to increase both the solubility and activity of the enzymes in organic solvents.

An entirely new approach is being developed for the enhancement of enzyme solubilization in organic solvents in the absence of water. The approach is to chemically bind enzymes to support molecules which are less polar using bridging chemicals that should not affect activity. This should render the enzymes more hydrophobic and more soluble in organics. The bridging chemicals are triazine dyes which are bound to polyethylene glycol (PEG).

Unbound alcohol dehydrogenase or hydrogen peroxidase in benzene showed no measurable activity, while both PEG-dye-enzyme complexes were active in benzene. In aqueous solution, the activity of alcohol dehydrogenase and laccase decreased with increasing PEG-dye concentration which indicates that the dye binding is competitive for the active site. On the other hand, peroxidase activity increased with PEG-dye concentration and cytochrome c was unaffected. In

further studies the solubility of cytochrome c in dry benzene was nearly 25 times higher when bound to the PEG-dye complex. In fact, when 1 mg of cytochrome c was bound to excess PEG-dye complex, dried and added to benzene, 80% of the cytochrome c was solubilized into the organic. The PEG-dye-enzyme complex method has general potential to enhance enzyme solubility and activity in organic media.

It has been suggested by several research groups to include a separations reagent in a fermentation reactor in order to isolate and concentrate the resulting product (often inhibiting to the fermentation). In conjunction with C. J. King and his coworkers at the University of California-Berkeley, we are initiating work on the production of organic acids by fluidized-bed bioreactors that also contain separations reagents for concentration of the product acid. The resulting four-phase system represents a somewhat more complicated control problem, but it should materially enhance the fermentation rate while reducing the total number of processing steps.

- (2) Multimembrane Bioreactor for Chemical Production (M. Shuler, Cornell University). The purpose of this project has been to test the feasibility of using a highly compartmented bioreactor for chemicals production using living cells in the presence of product inhibition and to provide a basis for rational scale-up of the reactor concept. Initial studies have focused on ethanol production from yeast. The reactor has consisted of four compartments separated by membranes. The gas layer is separated from the cell layer by a hydrophobic membrane; the cell layer is separated from the nutrient layer by a hydrophilic membrane; and the nutrient layer is separated from an extracting solvent (typically tributyl phosphate, TBP) by another hydrophobic membrane. The inclusion of the gas layer allows the efficient removal of CO₂ generated in the fermentation which has been a problem with most immobilized cell reactors. The hydrophilic membrane is used to entrap the yeast cells so as to increase effective cell density and volumetric productivity. The nutrient layer/solvent hydrophobic membrane allows the in situ extraction of ethanol into the TBP. By keeping the pressure of the nutrient layer higher than solvent layer, solvent emulsification can be prevented. Prevention of emulsification protects the cells from the toxic effects of TBP seen in shake flask cultures. Droplets of emulsified TBP can interact adversely with the yeast cell surface.

During the last year we have established that cycling the pressure of the gas layer can improve reactor performance, and this work was published. When operating the batch-recycle mode, pressure cycling decreases mass transfer limitations within the cell layer and across the hydrophilic membrane, thus making ethanol and glucose concentrations in the cell layer and nutrient layers approximately equal. The removal of mass transfer resistance increased reaction rate about five-fold.

We have attempted to operate this reactor system on a continuous basis. Initial experiments demonstrated continuous operation for up to 834 hours, but these runs were hampered by instability of the pressure cycle, largely due to wetting of the hydrophobic gas/cell membrane on the gas side. This membrane serves essentially as an overflow control device and is an essential component of a strategy of controlling the cycle based on time rather than actual volume exchanged. The membrane can become wet due to condensation or due to ethanol build-up in the cell layer which can then fill the membrane. The wet membrane will not allow the passage of gas, thus trapping CO₂ generated by fermenting cells. The trapped CO₂ excludes liquid nutrient from the cell layer starving the cells. Small perturbations in operating parameters can show positive feed-back and accumulatively can cause wetting. A vigorous gas flow to remove condensate on the membrane is helpful but does not solve the problem. In the initial continuous runs wetting occurred two or three times; the membranes could be cleaned with a hot water flush on the gas side but each wetting incident had an unfavorable effect on cell viability. A further implication of the membrane wetting problem has been an effective limitation on glucose input into the system, since high rates of CO₂ evolution aggravate the wetting problem.

A further problem in some, but not all, preliminary runs was leakage of cells from the hydrophilic membrane. Pressure cycling causes significant "bulging" of this membrane. Although catastrophic failures were not observed, cell leakage complicates interpretation of experimental data and also appears to lead to fouling of the hydrophobic membrane between nutrient and solvent layers, thereby leading to a decrease in extraction efficiency.

We have circumvented these problems by removing the gas/cell membrane and replacing it with a conductance liquid level control system. The new system directly

controls the pressure cycle through monitoring the actual volume exchanged and operates very stably. Also, stainless steel mesh screens have been added as further membrane supports. One currently active unit of this type has been operating for more than 1300 hours with nearly constant output parameters. With a feed concentration of 350 g/L glucose and about 80% conversion the ethanol volumetric productivity is about 5 g/L-h.

This system is most likely not near its optimal operating point. The addition of the stainless steel mesh has led to gas bubbles being entrapped on the underside of the cell/nutrient hydrophilic membrane and thus decreasing the efficiency of pressure cycle operation. We are exploring operating the system with the reactor oriented vertically rather than horizontally, which should greatly reduce problems of gas trapping.

In addition to the demonstration of long-term continuous operation, we have completed in the last year a detailed computer model for *S. cerevisiae*. This cellular model can be coupled to models of both batch reactors with suspended cells and to a model of the multi-membrane reactor system itself. The combined models predict dynamic performance including the effects of pressure cycling. This model should greatly aid scale-up.

- (3) Biocatalyzed Hydroxylation in Organic Solvents (A.M. Klibanov, Massachusetts Institute of Technology). During this year, we have extended our studies of enzymes in non-aqueous media from enzymatic catalysis (especially, that by oxidative enzymes) in organic solvents to enzyme-catalyzed gas phase reactions, i.e., in the absence of any liquid phase. Using yeast alcohol oxidase as a model, we have demonstrated that dehydrated preparations of the enzyme adsorbed on DEAE-cellulose (and other supports) vigorously catalyze a gas phase oxidation of ethanol vapors with molecular oxygen. The enzymatic activity in the system is severely inhibited by the product hydrogen peroxide. This inhibition can be alleviated, however, by an addition of catalase or peroxidase to the dry preparation. Such dehydrated bi-enzyme catalysts afford a complete and selective conversion of the substrate to acetaldehyde.

Mechanistic investigation has revealed that the water activity is a critical parameter in the enzymatic

conversion of gaseous substrates. Enzymatic activity in the above-described gas-phase reaction increases several orders of magnitude, whereas the thermostability decreases drastically, when the water activity is increased from 0.11 to 0.97. Alcohol oxidase is catalytically active on gaseous substrates even at hydration levels below the monolayer coverage. The enhanced thermostability at lower hydrations results in an increase in the temperature optimum of the gas phase reaction catalyzed by alcohol oxidase. The apparent activation energy decreases as the water activity increases, approaching the value obtained for the enzyme in aqueous solution. The formation of a preadsorbed ethanol phase on the surface of the support is not a prerequisite for the enzymatic reaction, suggesting that the reaction occurs by direct interaction of the gaseous substrate with the immobilized enzyme. The gas phase enzymatic oxidation of ethanol follows Michaelis-Menten kinetics, with a K_m value almost 100 times lower than that in aqueous solution. Based on vapor-liquid equilibrium data and the observed Michaelis constant values, it is postulated that during the gas phase reaction the ethanol on the enzyme establishes an equilibrium with the ethanol vapor similar to that between ethanol in water and ethanol in the gaseous phase. Direct comparison shows that the optimal enzymatic activity of alcohol oxidase in the gas phase is comparable to that in water.

The data obtained may be useful for the rational design of novel gas-solid bioreactors. Also, we have developed an enzyme-based device for the determination of analytes in the gaseous phase. This approach has been successfully tested for qualitative and quantitative enzymatic measurements of ethanol in the air.

- (4) An Integrated Biological-Chemical Process (Tsao, Purdue University). The objective of the project is to couple a fermentation step with a product separation step. The overall integrated process can then be modeled and optimized as a whole. The 2, 3-butanediol fermentation and its separation are selected as the model system for investigation; the concept is, however, generic in nature.

The work in the past ten months is preliminary and lays the foundation for the project. We have selected a packed column and a fluidized bed containing beads of immobilized microbial cells as the reactor. Work has been successfully carried out to immobilize Klebsiella

oxytoca in calcium alginate beads. We designed and constructed locally a simple device with an air jet and a syringe controlled by a precision pump to make the beads of uniform sizes.

The beads have been tested for butanediol production giving, however, only a low product yield. This could be due to oxygen limitation because even though the Klebsiella oxytoca's fermentation is anaerobic, a controlled supply of oxygen will enhance the diol yield at the expense of by- or co-product. A parallel study was carried out applying nitrate as a possible source of oxygen in addition to air. The nitrate was found to be useful.

The low yield in alginate beads could also be due to the low level of phosphate addition (for avoiding calcium phosphate precipitation). We plan now to use other immobilized carriers to replace alginate if the phosphate is indeed limiting.

Meanwhile, a systematic study is under way to model the kinetics of diol formation and cell growth. The model is based on bioenergetic considerations as well as carbon balances. Pyruvic acid plays a key role in the model. When it is converted into a number of possible metabolic products, a varying level of energy is needed. The energy need is met by the energy generating steps in the overall metabolic activity. The model has been found to agree with the experimental data, at least qualitatively. A new instrument setup has just been put together. We are ready for the next phase of systematic experimental data collection to estimate the parameters in the model.

Adsorption by insoluble resins or activated carbon is selected as the method for the isolation of the diol product. We tested the adsorption of diol product by a resin containing pyridine and found good selectivity. We also tested the adsorption of butanediol by zeolite with satisfying results. The rate of adsorption is fast. The adsorption isotherms are being determined.

In brief, in the past ten months we made substantial progress in examining individual process steps of the intended overall coupled fermentation and separation process. We are more or less ready for integrating the steps. The modeling work will give us the necessary mathematical tools for simulation and optimization of the overall process.

- (5) Separation by Reversible Chemical Association (C.J. King, University of California, Berkeley). Reversible chemical association, or complexation, is a promising method for recovery of carboxylic acids, alcohols, glycols and related substances from the dilute solutions resulting from manufacture of these chemicals by fermentation of biomass. Association reactions offer selectivity among solutes and high capacities at high solute dilution. Both these properties fit separation needs in bioprocessing.

We have focussed upon use of association reactions in extraction and sorption, although separations by complexation can also be implemented in other ways, such as membrane processes, crystallization, and extractive distillation.

We have measured equilibria for extraction of acetic, succinic, malonic, lactic, maleic and fumaric acids from aqueous solution by Alamine 336 (Henkel Corp.; tri-C₈ to C₁₀ amine) in several diluents, including heptane, methyl isobutyl ketone (MiBK), chloroform, methylene chloride and nitrobenzene. The association equilibrium constant for the 1:1 complex depends strongly upon both the acidity (pK_a) of the acid and the chemical nature of the diluent. Stoichiometric overloading (more than one acid extracted per amine) occurs in a number of cases. Overloading tendencies are also strongly affected by the chemical nature of the diluent, and are greater for monocarboxylic than for dicarboxylic acids.

We have rationalized these equilibria through mass-action-law modeling, in light of relevant spectroscopic measurements and comparable results from prior studies. The indicated association constants have been related to chemical parameters. From measurements at different temperatures, enthalpies and entropies of complexation of lactic and succinic acids by Alamine 336 in chloroform and MiBK diluents have been derived and rationalized. Preliminary measurements have been made of the amount of water co-extracted with the carboxylic acid.

We have obtained and interpreted exploratory data characterizing regeneration of amine extracts by each of four different methods: (i) Back-extraction into water following a change in temperature. (ii) Back-extraction into water following a change in diluent composition. (This approach can be combined with i). (iii) Precipitation of a low-solubility acid (e.g., fumaric) following evaporation of diluent and/or

temperature change. (iv) Back-extraction into an aqueous solution of a volatile tertiary amine (e.g., trimethylamine), followed by concentration and decomposition of the trialkylammonium carboxylate to produce the product acid and the light amine for recycle. The tertiary amine is needed since primary and secondary amines, and ammonia, can form amides.

Future research will explore and evaluate the various methods of regeneration more deeply, as well as investigating alcohol and phenol diluents and competitive equilibria in the presence of multiple acids.

We have measured and interpreted equilibrium uptakes of acetic acid and water from aqueous solution by various functionalized basic polymeric sorbents. We have also measured and interpreted equilibria for regeneration by leaching of sorbed acetic acid from sorbents with different degrees of basicity, with solvents having various degrees of basicity.

Most basic sorbents are difficult to regenerate; however we have demonstrated the feasibilities of regenerating Dowex MWA-1 tertiary amine resin (Dow Chemical Co.) by hot-air stripping; regenerating Aurozez polybenzimidazole resin (Celanese Chemical Co.) by leaching with successively less polar solvents; and regenerating polyvinylpyridine sorbents by leaching with common solvents. The polyvinylpyridine sorbents swell considerably, however.

We have found that phenols interact more strongly with alcohols than do other common solvents. m-Cresol provides an equilibrium distribution ratio (D) of 2.2 for extraction of ethanol from water, whereas the best solvents in other classes that have been tested provide values of D in the range 0.5 to 1.0.

Through measurements of extraction of ethanol and each of the four butanol isomers by m-cresol in various diluents, we have shown that specific 1:1 alcohol:cresol complexes are formed.

Future research will address extraction of glycols, use of sorbents with phenolic groups, other functional groups effective for complexing of alcohols and phenols, and the possibility of recovery of glycols through adductive crystallization.

Sufficiently non-polar polymeric adsorbents, such as styrene-divinylbenzene (Rohm & Haas Amberlites XAD-2

and XAD-4) and pyrolyzed sulfonated styrene-divinylbenzene copolymers (Rohm & Haas Ambersorbs), are not wetted by dilute aqueous solutions. We have found that, in the non-wet state, such adsorbents take up solutes of moderate and high volatility at adequate capacities and substantial rates. The non-wet adsorbents provide advantages of low concomitant water uptake and an ability to fractionate among solutes on the basis of volatility.

Measurements of adsorption of acetic and propionic acids, ethanol, succinic acid and 1,3-butanediol have demonstrated that the rate limit is Knudsen diffusion through pores, and that performance of fixed-bed adsorbers can be rationalized through that concept.

Future work will explore the use of these non-wet adsorbents at higher temperatures, which should give economically attractive sorption rates for glycols.

- (6) Enzyme Catalysis in Non-aqueous Solvents (H.W. Blanch, University of California, Berkeley). This research program has been concerned with developing techniques which will permit the wider use of enzymes in the synthesis of organic chemicals. Although enzyme catalysis offers unique features, such as stereo and regiospecificity, and the ability to conduct the reactions at ambient temperatures and pressures, it has not found widespread use in the chemical process industry, due primarily to the low aqueous solubility of many potential reaction substrates of interest. In order to increase the range of possible substrates, the research has examined the use of two-phase aqueous-organic systems, where the enzyme is maintained in an aqueous milieu and the substrate solubility is enhanced in the second organic phase. Several approaches have been examined, including the use of liquid membrane systems, reverse micelles and microcapsules. Two model enzyme reactions have been studied: the production of tryptophan from indole and the oxidation of the steroid cholesterol.

Tryptophan production has been studied in liquid membranes, where the enzyme is retained within an inner aqueous phase surrounded by a thin spherical layer of a second organic phase. This is then placed in a second aqueous phase. By selecting appropriate phase-transfer catalysts (eg., liquid ion exchangers), substrates and products can be partitioned from one phase to the other. The second system examined for tryptophan production was the use of reverse micelles. A micelle is formed upon the addition of a small volume of water

to a much larger volume of immiscible organic solvent containing a surface active agent. The surfactant is oriented at the interface of the two phases so that the hydrophilic head is in contact with the aqueous phase and the hydrophobic tail is in the bulk organic phase. Micelles are thus usually envisioned as spherical clusters of surfactant surrounding an inner pool of water, which is capable of solubilizing large hydrophilic molecules such as proteins. An enzyme solubilized in the inner water pool is thus protected from direct contact with the surrounding organic phase. Favorable partition coefficients for the enzyme substrates and products can change the equilibrium concentrations by several orders of magnitude, due to the driving force created by the free energy change of the transfer of a reagent from one phase to another. This approach to enzyme catalysis in organic solvents thus appears most attractive and of considerable generality.

Cholesterol is only sparingly soluble in water and its enzymatic conversion to 4-cholest-3-one proceeds at low rates. The use of a second liquid phase to serve as a reservoir of cholesterol has been examined. An aqueous dispersed phase containing enzyme is contacted with the organic phase and cholesterol is transferred to the enzyme and product preferentially partitions from the aqueous to the organic phase. While this approach is the simplest, the possibility of denaturation of the enzyme at the interface and inhibition by solvent do not make this approach attractive. For these reasons, we have examined encapsulation as a method for protecting the enzyme, overcoming some of the problems associated with the fragility of micelles and providing a means for developing a fluidized bed bioreactor based on encapsulated enzymes. In addition, microencapsulation provides a promising approach for developing a robust method for cofactor recycle.

Tryptophan production was observed in both a reverse micelle reactor and a supported liquid membrane. A quaternary alkyl amine was successfully employed in both systems to transport tryptophan to the organic phase.

Tryptophan was produced from indole and serine in a micellar solution of Brij 56 in cyclohexane. Aliquat-336 was successfully employed for transporting the amino acids between the water pool and bulk organic phase. The presence of the anion exchanger placed certain constraints on the choice of the surfactant and

aqueous phase buffer. The productivity of this system base was found to be 10 g Trp/g catalyst·L·hr. The productivity of the micellar reactor based on initial rate data was greater than the productivity of all other systems reported in literature.

Experiments employing a supported liquid membrane were designed chiefly to study the transport of tryptophan through the organic phase. This is the key factor in tryptophan production in a liquid-membrane emulsion-type reactor. The production of tryptophan in a such a system will be limited by the maximum solubility of the enzyme and its stability within the inner aqueous phase. The transport of ammonia and pyruvate (or serine) to the reaction phase and the maintenance of a differential in pH between the two aqueous phases are also of concern. The primary advantage of the emulsion system over the micellar system is the continuous extraction of tryptophan into a second aqueous phase, simplifying product recovery.

The use of liquid membranes has been demonstrated to be an efficient means of extracting tryptophan from an aqueous reaction phase. The organic phase acts as a selective barrier and also serves as a reservoir of indole. It is possible to maintain a differential in pH between the two aqueous phases, provided that appropriate buffers are chosen. A lower pH in the stripping phase increases the rate of transport of tryptophan by allowing the equilibrium concentration in the stripping phase to greatly exceed that in the reaction phase.

An effective diffusivity of 4.2×10^{-6} cm²/sec for the tryptophan Aliquat complex at 37°C was estimated by correlating the data to a mathematical model. The model also predicts that high rates of tryptophan transport may be observed in a liquid membrane emulsion-type reactor. The transfer of ammonia to the reaction phase and the stability of the enzyme in such an environment are the key factors for the successful production of tryptophan in a liquid membrane.

The analysis of equilibrium data in two-phase systems has been examined as part of the broader objectives of the sponsored research. The thermodynamic analysis of such systems has not been dealt with in a consistent manner in the literature.

The presence of the organic phase in a two-phase system facilitates a shift in equilibrium by supplying substrates and by extracting products until both

reaction and phase equilibria have been achieved. The magnitude of the shift is dependent on the partition coefficients of the reacting species and the volume ratio of the two phases. Reactions with hydrophobic products are most appropriate for two-phase systems. Solvent selection is critical since the partition coefficients are unique for each solvent.

The oxidation of cholesterol to 4-cholesten-3-one by cholesterol oxidase has been examined in both a two-phase aqueous-organic system and in microcapsules containing the enzyme in an aqueous environment in a second organic phase. In the two-phase system, the aqueous enzyme phase is dispersed in the cholesterol rich organic phase. The kinetics of the oxidation reaction have been examined in detail for this system, as the coupling of mass transfer and reaction is common to other systems of this type and to date no rational analysis of this system has been available. The effect of enzyme concentration, oxygen concentration (a co-substrate in the reaction), cholesterol concentration and the effect of various surfactants has been examined. From the kinetic data it is apparent that the system operates in a regime where the coupling of cholesterol transport through the inner aqueous film and reaction kinetics is important. The characteristic time constants for reaction are greater than that for diffusion and thus the operative regime is the slow reaction regime. The system operates in the transition between kinetic and diffusion control with the slow reaction regime. In this situation, interfacial area, kinetics and substrate concentration are all important in determining the observed rate.

The possibility of interfacial activity of cholesterol oxidase was examined. Interfacial tension measurements at the organic-aqueous interface determined the surface excess of cholesterol. A simplified model of the kinetics of an enzyme reaction at the interface was developed and used to compare the observed kinetics. It appears that although cholesterol oxidase could be active at the interface, some solution activity is indicated. It is not possible to distinguish between the slow reaction regime and interfacial enzyme activity.

As part of this project, new techniques for the determination of cholesterol concentration in solution were examined. A method for analysis of cholesterol in non-aqueous solutions employing a cholesterol oxidase-peroxidase coupled bienzymatic system was reported by Kazandjian, Dordick and Klibanov. This was examined

in detail and the method was found to depend on the nature of the environment in which the peroxidase is employed. The method suggested by the above authors employed enzyme immobilized to glass beads. This was found to influence the nature of the product formed and a more reliable approach was developed. It involves a two-phase system with enzymes contained within an aqueous dispersed phase. This method yields consistent results over a wide range of substrate concentrations and is insensitive to factors such as enzyme attachment to the containing vessel, which caused difficulties with the other method. The details of this new method, which would be applicable to a variety of substrates only sparingly soluble in water, have been recently published.

Another approach to enzymatic conversion of sparingly soluble substrates was examined. A method of interfacial polymerization was developed to encapsulate enzymes in an aqueous miniphase. This approach has the benefits of providing an aqueous environment for the enzyme, yet solubilizing substrates in a continuous organic phase. Direct contact of the enzyme and organic is thus prevented and the microcapsule serves as a semipermeable membrane permitting solute transport but retaining enzyme and water-soluble cofactors within the capsule. This approach is attractive for cofactor regeneration as two enzymes can be coimmobilized within the capsule and two substrates consumed simultaneously to produce product and regenerate the required cofactor. Initial studies employed cholesterol oxidase as a model system, and NAD⁺ cofactor regeneration schemes have been examined in a second model system, alcohol dehydrogenase. Stable operation of the cofactor recycle scheme using the cinnamyl alcohol/cinnamaldehyde pair has been demonstrated and the microcapsule system has shown stable operation in a fluidized bed for periods of greater than 10 days.

Microcapsules are relatively fragile and new chemistries have been examined to provide enhanced mechanical stability while retaining desired porosity. Interfacial polymerization has been found to be the most effective method for obtaining the desired balance of strength and permeability. Several problems inherent with this approach have been overcome - the pH of the interfacial polymerization reaction results in intracapsule pH values of ~9, and the pH must be adjusted by proton exchange to near neutrality for most enzymes. Quaternary alkylamines were found to be effective for this purpose, permitting pH alteration within the capsules suspended in an organic phase.

^{31}P NMR was used to determine intracapsule pH and confirm the optimum pH for cholesterol oxidase under these conditions. Triethylphosphate was used as internal standard for calibration of the NMR signal. This technique has excellent potential for non-invasive measurement of substrate and reactant concentrations within capsules.

- (7) Multiphase Fluidized Bed Bioreactor (B. Allen, Battelle Memorial Institute). The goal of this program was to develop a continuous Multi-Phase Fluidized Bed (MPFB) bioreactor by establishing an experimental and design data base which can be used to ultimately commercialize the MPFB for the bioproduction of chemicals. The program accomplishments and major findings are described below. Active and stable biocatalyst calcium alginate beads containing cells of the bacterium Clostridium acetobutylicum were obtained. The ability to scale up the production of these beads to meet the needs of a large experimental scale fluidized bed was accomplished. It was also demonstrated that the density of the gel biocatalyst beads is a function of the liquid medium in which the beads are equilibrated.

Two butanol extractants, oleyl alcohol and trioctylphosphine oxide (TOPO) were verified to have sufficiently high distribution coefficients for butanol. They were non-toxic for growth and product formation for cultures of Clostridium acetobutylicum. They will be suitable for demonstration in the MPFB bioreactor.

Design data on the MPFB bioreactor revealed that axial mixing with these alginate beads, even at very low Reynolds number, is large. The degree of axial mixing would likely increase with the introduction of gases emanating from an actual bacterial fermentation and when scaling up to larger column diameters (i.e., lower height-to-diameter ratios). Back mixing might become a problem in larger reactors. The use of Laser Doppler Velocimetry was useful for measurements of liquid and particle flow velocities within a cm of the measuring tip. This method will have application in characterizing fluidized bed flow but does have limitations. The evaluation of mass transfer coefficients in fluidized beds was begun but will require the development of kinetic models and the obtaining of actual fermentation data.

The initial proof-of-concept tests on the ability of the MPFB reactor to extract butanol at rates

comparable to that expected in an actual fermentation were obtained. A constant ratio of 4.23 for butanol in oleyl alcohol versus butanol in water in an extraction test in the MPFB reactor was obtained (no biocatalysts were employed, but butanol, acetone and ethanol were fed into the reactor at rates mimicking an actual fermentation). Similar constant ratios were observed for acetone and ethanol, e.g., 0.44 and 0.29 respectively. These tests verified the basic principle of the MPFB bioreactor, that in situ extraction could occur thus controlling product inhibition.

An independent technical/economic analysis was conducted of the MPFB process for butanol. The major conclusion from the analysis was that the MPFB process could potentially, when fully developed and scaled up, reduce the cost of butanol production by fermentation from well over \$1.00/lb to \$0.59/lb or less. The study also indicated that flow rate ratios (distribution coefficients during operation) must be of the order of 2 to 3 to achieve major reductions in the production cost of butanol with available extractants.

Since the MPFB bioreactor was able to easily operate with extractant-to-aqueous phase flow rate ratios as high as 4 to 1, the ability to lower process costs appears quite feasible. If this property of the fluidized bed is maintained during actual operation with live biocatalyst beads, then a significant step will have been taken towards developing a bioreactor capable of continuous production. Efforts that integrate the technical elements of the work performed up to this point into an actual, working MPFB bioreactor are the focus of our current efforts.

The results from the fluid dynamics studies also revealed additional research areas that will need to be addressed in the future. The importance of axial mixing in the evaluation of bioreactor performance, modeling and scale-up is often overlooked in bench scale studies. More attention will need to be given to this operational parameter especially in experiments involving biocatalyst. Careful attention will need to be given to the back mixing caused by gas evolution and the apparent increase in axial mixing when scaling factors are determined since such factors may be non-linear. This data also implies that laboratory kinetic investigations should potentially be run in back-mixed systems, not tubular reactors with high L/D (length/diameter) ratios. The use of gel biocatalyst beads in a fluidized bed reactor presents a more

complex situation from a modeling standpoint than is encountered with more conventional catalyst supports. Since the density of the biocatalyst beads could be variable this variable must be considered in evaluating reactor performance. Finally, since the fluidized beds of biocatalyst gel beads operate in Reynolds number ranges normally associated with fixed beds, additional work is needed to properly characterize the hydrodynamics and mass transfer coefficients of these systems. The use of an extractant with high distribution coefficients would assist in improving even further process costs. One extractant, liquid phosphine oxide, which is very similar to TOPO, would be an attractive candidate if the specific toxic component of its mixture (if indeed, one exists) could be identified and removed without impairing the distribution coefficient greatly. This or other extractants would need to be tested and evaluated for possible use in larger scale processes.

C. Process Design and Analysis

- (1) Bioprocess Synthesis, Integration, and Analysis (J. D. Ingham, Jet Propulsion Laboratory). The purpose of this task is to develop computer modeling and bioprocess simulation methods for rapid, realistic assessments of new bioprocess concepts, and to synthesize and evaluate a series of candidate bioprocesses for added-value commodity chemicals to determine energy requirements and economics, and the potential for process technology transfer and commercial development. Bioprocess models will also be developed to confirm potential improvements and advantages of new biocatalyzed processes, research directions and achievements, and provide guidance in proposal evaluation and the selection of future research tasks.

The technical approach includes: development of energy-economic computer models for the assessment of biocatalyzed processes and comparison with petrochemical processes, and synthesis and evaluation of promising biocatalyzed processes for the production of commodity chemicals. These bioprocesses will be modified to include projected research advances (e.g., genetically engineered microorganisms, bioreactor modeling and verification, membrane development, and biocatalyst immobilization) for energy-economic comparative assessments. Four parameters that significantly affect bioprocess economics and total energy requirements are cell density, productivity, product recovery energy and yield.

Large-scale production of commodity chemicals by biocatalyzed processes (where cellulose, starch or hexose feedstocks may be utilized) could significantly decrease U.S. dependence on the supply of foreign and domestic nonrenewable petroleum resources, but because of relatively high feedstock costs and some specific limitations of biocatalyzed processes, most of them are not yet competitive with current petrochemical routes, especially with respect to process economics. Industrial ethanol is an exception, and is produced in large quantities (annual US production: >5 billion lb) by fermentation and by hydration of ethylene produced by high-temperature catalytic cracking of petroleum products, but interest in increased plant capacity for fermentation has subsided as the cost of petroleum has decreased.

The characteristics of biocatalyzed processes often result in relatively low rates of reaction and production, and low product concentration (normally in water). Extensive assessments of a series of processes for ethanol (as a model of a high-volume industrial chemical) have been completed to establish a more quantitative basis for estimation of the relative effects of production rates and product concentration in the bioreactor on process energy and economics. The results of model bioprocess simulations for ethanol show that energy consumption is sensitive to product concentration, is essentially independent of rate of production, and that the effect of production rate on economics is at least an order of magnitude less than the effect of product concentration. The calculations also suggest that concentration is likely to be even more important for other potential industrial chemical biocatalyzed processes, e.g., for n-butanol or acetic acid, where product recovery is more energy intensive than for ethanol, because n-butanol is produced at low concentrations, and acetic acid is difficult to separate from water. It has been concluded that future research advances to improve the energy, economics and effective synthesis of new, competitive biocatalyzed processes for industrial chemicals should be directed toward significantly increasing product concentration, rather than attempting to independently maximize only rates of production. A paper describing this work has been completed and was published in the ECUT Program Bulletin, Jan-Feb, 1988.

Computer programs have also been developed that can be used to model bioreactors, where specific parameters characteristic of the microorganism are used to derive bioprocess productivity, conversion, and product yields

and concentration. These can be applied to the evaluation of bioreactor configurations that should result in lower energy requirements and product costs. The bioprocess models, (where fundamental kinetic equations and intrinsic parameters, e.g., maximum specific rates, saturation constants, and yield factors, result in fixed relationships with respect to conversion, product yield, product concentration, and productivity) have been applied to show that there are maximum limits for these parameters for specific bioprocesses. For example, the highest productivities normally will occur at relatively low yields and product concentrations and corresponding high costs of production. Calculations for simulated processes with variations in rate, yield, and concentration agree with previous experimental observations, and show that bioreactor systems that can be operated continuously at high cell concentrations result in the highest yields and rates of production.

Although inhibition of the rate of product formation can be minimized by removal of the product as formed (as by extraction or vacuum vaporization), evaluations of ethanol and n-butanol bioprocesses show that the increase in rate is usually less than a factor of two, but higher conversions can usually be obtained because the upper limit for rate inhibition is never reached. The rate limitation (of a factor of about two) arises because even low-productivity fermenters are generally not operated much beyond the point where the actual average rate has been inhibited to the extent that it is decreased to less than about 50% of the intrinsic maximum for the microorganism, i.e., the maximum specific rate, as mass of product/mass of cells/hour. Since rates are approximately proportional to cell concentration, more pronounced rate increases (by a factor of four or more) can be obtained in continuous processes where cell concentration is maximized, e.g., by cell recycling or use of a tower fermenter. For product extraction with the solvent in intimate contact with the cells, the rate of production may still exceed the rate of extraction; therefore, the separation rate does not seem to be significantly increased by close contact with cells. Since extractant toxic effects are increased and are usually a serious problem, extraction effectiveness can be improved by multistage extraction after cell separation; for example, by downstream treatment of tower reactor effluent, or by extraction through a membrane.

Ethylene is the leading commodity chemical in the U.S. (33 billion lb produced in 1986), and is currently

produced from fermentation ethanol in some countries where petroleum resources are less readily available. At present hydration of petrochemical ethylene to ethanol is economically competitive with fermentation ethanol, but at very high energy cost (>28,000 btu/lb ethanol, total process energy only, compared to <4000 btu/lb for bioprocesses). In potential processes where bioprocess ethanol can be substituted for ethylene without direct dehydration to produce a derivative chemical, total energy required would be much less, and any economic advantage will depend on the value added. The greatest advantage of such processes would probably be in the utilization of renewable biomass and corresponding conservation of petroleum resources. For two candidate products, ethyl acetate and acetaldehyde, the value increase exceeds 100%, relative to ethanol; therefore, work has been initiated to synthesize advanced processes and model utilization of bioprocess ethanol for chemical production of acetate ester and aldehyde. Costs of production and energy required (relative to corresponding petrochemical processes) will be compared. The potential advantages depend partly on successful design, integration and recovery of process energy, e.g., optimization of heat-exchanger networks for overall processes.

In addition to process synthesis and assessments for ethyl acetate and acetaldehyde, future plans for FY 1989 include modification and utilization of a commercial simulation program (e.g., a version of ASPEN) in conjunction with JPL programs for energy-economic assessments and for comparison of alternative bioprocess technology with relevant petrochemical production.

- (2) Assessment of the Role of Biotechnology in Commodity Chemical Production (K.M. Stern, Chem Systems Inc.). The goal of this task (which was completed in FY 1988) was to evaluate the current perspectives of major commodity chemical producers and biotechnology-oriented companies toward the goal of bioproduction of commodity chemicals, and to identify the key issues that these companies see as influencing potential industrial applications of bioprocesses.

The approach has been to determine which relevant companies would be interested in attendance and participation in a roundtable conference on bioprocess chemical production; compile a list of candidate chemicals and select the most promising; and establish the basis for process evaluations (in cooperation with participating companies), followed by technoeconomic

assessments of bioprocesses for selected chemicals. Then a conference was convened to arrive at an industry position on the feasibility of increased commodity chemical production by bioprocesses, including identification of any major technical or economic obstacles to expanding such applications.

The chemicals selected for assessments were propylene oxide and caprolactam. It was concluded that an Exxon bioprocess for propylene oxide could be economically competitive with current commercial processes if it can be demonstrated at a reasonable scale of operation. The process is based on the use of immobilized methylotropic microorganisms to enzymatically catalyze the oxidation of propylene to the epoxide, but the process also requires methane and methanol for cell production and regeneration, and is very energy intensive.

Similar results were obtained in assessments of processes for caprolactam. Adiponitrile (which can be made by reaction of butadiene with hydrogen cyanide) is enzymatically converted to 5-cyanopentanoic acid which is then reacted with hydrogen to make 6-aminocaproic acid, which can be dehydrated to epsilon-caprolactam. This process should be economically competitive after demonstration at a reasonable scale, but energy required is higher than for the current commercial process.

On June 21-22, 1988, an industry discussion group was convened in Washington D.C. to consider the role of biotechnology in U.S. commodity chemical production. Some major comments at this meeting suggest that for production of commodity chemicals by biocatalyzed processes, expectations have been limited by inadequate separation technology and biocatalyst stability, and institutional inertia to retain conventional chemical processes. Recommendations for future work included: improvements in separation processes, biocatalyst stability, cofactor regeneration, emphasis on enzymes, analysis of electrically conductive polymers as electron sinks, development of biomimetic systems, more active publication and technology transfer efforts, and increased development and enhancement of assessment methods for more meaningful comparisons of process economics.

Representatives from the Department of Energy, Celgene, AMGEN, Genex, Dow Chemical, PPG Industries, E.I. Du Pont and Eastman Kodak attended the meeting, in

addition to those from the Jet Propulsion Laboratory
and Chem Systems.

SECTION IV

PUBLICATIONS

Allison, J.M. and W.A. Goddard. 1985. Active Sites on Molybdate Surfaces, Mechanistic Considerations for Selective Oxidation, and Ammoxidation of Propene. ACS Symposium Series No. 279:23-36.

Allison, J.M. and W.A. Goddard. 1985. Oxidative Dehydrogenation of Methanol to Formaldehyde. J. Catal. 92:127-135.

Arnold, F.H.. 1988. NMR Studies of Crambin Structure and Unfolding in Nonaqueous Solvents. National Acad. of Sci. (in press).

Arnold, F.H.. 1987. Protein Engineering for Nonaqueous Solvents. Protein Engineering 2:21-15.

Bailey, J.E.. 1987. Genetically Structured Models for Growth and Product Formation in Recombinant Microbes. In: Horizons of Biochemical Engineering, University of Tokyo Press, Tokyo, p.77.

Bailey, J.E., M. Hjortso, S.B. Lee and F. Srienc. 1983. Kinetics of Product Formation and Plasmid Segregation in Recombinant Microbial Populations. Annals of the New York Academy of Sciences. "Biochemical Engineering III," 413:71.

Bailey, J.E., N. Da Silva, S.M. Peretti, J.-H. Seo, and F. Srienc. 1986. Studies of Host-Plasmid Interactions in Recombinant Microorganisms. Annals N.Y. Acad. Sci. 469:194.

Bailey, J.E., D.D. Axe, P.M. Doran, J.L. Galazzo, K.F. Reardon, A. Seressiotis, and J.V. Shanks. 1987. Redirection of Cellular Metabolism: Analysis and Synthesis. Annals New York Acad. Sci. 506:1.

Baresi, L. and M. Dastoor. 1987. The ECUT Biocatalysis Project. In: Energy Conversion and Utilization Technologies: A Program Bulletin.

Barzana, E., A.M. Klibanov and M. Karel. 1987. Enzyme-catalyzed Gas Phase Reactions. Appl. Biochem. Biotechnol. 15:25-34.

Bertani, L.E. and G. Bertani. 1985. Chromosomal Integration and Excision of a Multicopy Plasmid Joined to Phage Determinants for Site Specific Recombination. In: Plasmids in Bacteria, D.R. Helinski, ed., Plenum Press, New York, p. 917.

Bertani, L.E. and G. Bertani. 1984. Proceedings of International Research Conference on Plasmids in Bacteria (Abstract); University of Illinois, Urbana.

Bertani, L.E. and E.W. Six. 1988. The P2-Like Phages and their Parasite, P4. In: The Viruses, editors: Wagner, R.R. and H. Fraenkel-Conrad, Plenum Publishing Corp.

Bioaspen. 1986. System for Technology Assessment, Technical Progress Report, Washington University, St. Louis, MO.

Biocatalysis Research Activity Annual Report: FY 1983, Jet Propulsion Laboratory, JPL Publication 84-27, DOE/CS-66001-4, April 15, 1984.

Biocatalysis Project Annual Report: FY 1985, Jet Propulsion Laboratory, JPL Publication 86-32, DOE/CS-66001-9, July 30, 1986.

Biocatalysis Project Annual Report: FY 1984, Jet Propulsion Laboratory, JPL Publication 85-31, DOE/CS-66001-8, April 15, 1985.

Biocatalysis Project Annual Report: FY 1987, Jet Propulsion Laboratory, JPL Publication 88-8, DOE/CS-66001-11, March 30, 1988. editor L. Baresi.

Biocatalysis Project Annual Report: FY 1986, Jet Propulsion Laboratory, JPL Publication 87-11, DOE/CS-66001-10, April 30, 1986.

Busche R.M. (1987). The Biomass Alternative. Bio En-Gene-Er Assoc. Inc.

Celniker, S.E., K. Sweder, F. Srienc, J.E. Bailey and J.L. Campbell. 1984. Deletion Mutations Affecting Autonomously Replicating Sequence of ARS1 of Saccharomyces cerevisiae. Mol. Cell. Biol. 4:2455.

Chem Systems, Inc.. 1984. Technical and Economic Assessment of Processes for the Production of Butanol and Acetone - Phase Two: Analysis of Research Advances. JPL 9950-928, DOE/CS-66001-5, Prepared for Jet Propulsion Laboratory by Chem Systems, Inc., Tarrytown, New York, August.

Chem Systems Inc.. 1986. Techno-Economic Assessment of Microbial Ammonia Production. Prepared for Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, New York, February.

Chem Systems Inc.. 1986. Assessment of ORNL Immobilized Cell Ethanol Fermentation Process. Prepared for Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, New York, March.

Chem Systems Inc.. 1986. Implications of ORNL Immobilization Technology on Citric Acid Production. Prepared for Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, New York, June.

Chem Systems Inc.. 1988. Assessment of the Likely Role of Biotechnology in Commodity Chemical Production. Prepared for the Jet Propulsion Laboratory by Chem Systems Inc., Tarrytown, NY, October.

Chemical Processes Project Annual Report: FY 1982, Jet Propulsion Laboratory, DOE/CS-66001-1 July (1983).

Chemical Process Research and Development Program, FY 1985 Annual Report, Lawrence Berkeley Laboratory, Applied Sciences Division, University of California, Berkeley, August, 1986.

Chen, G. and W.A. Goddard III. 1988. The Magnon Pairing Mechanism of Superconductivity in Cuprate Ceramics. Science 239:899-902.

Cho, T.Y. and M.L. Shuler. 1986. Multi-Membrane Bioreactor for Extractive Fermentation. Biotech. Prog. 2:53-60.

Cho, T.Y.. 1987. Ph.D. Dissertation, Multimembrane Bioreactor for Extractive Fermentation: Ethanol from Saccharomyces cerevisiae. Cornell University.

Davison, B.H. and C.D. Scott. 1986. Ethanol Production on an Industrial Feedstock by Zymomonas mobilis in a Fluidized Bed Bioreactor. Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May 13-16.

Davison, B.H.. (1988). Mixing and Dispersion in a Columnar Three-Phase Fluidized-Bed Reactor. 10th Symposium on Biotechnology for Fuels and Chemicals.

Davison, B.H. and C.D. Scott. 1986. Fermentation Kinetics of Zymomonas mobilis under Mass Transfer Limitations. 192nd ACS National Meeting, Anaheim, CA, September 7-12.

Detroy, R.. 1987. Report on Biocatalysis in Organic Media. Conference in The Netherlands.

DiStefano, S., A. Gupta and J.D. Ingham. 1983. Generation of Chemical Intermediates by Oxidative Decarboxylation of Organic Acids. JPL Publication 83-19.

Dordick, J.S., M.A. Marletta, and A.M. Klivanov. 1987. Peroxidases Depolymerize Lignin in Organic Media but not in Water. Proc. Natl. Acad. Sci. USA 83:6255-6257.

Dordick, J.S., M.A. Marletta, and A.M. Klivanov. 1987. Polymerization of Phenols Catalyzed by Peroxidase in Non-Aqueous Media. Biotechnol. Bioeng. 30:917-920.

Efthymiou, G. S. and M. L. Shuler. 1987. Elimination of Diffusional Limitations in a Membrane Entrapped Cell Reactor by Pressure Cycling. *Biotechnology Progress* 3:259-264.

Eggers, D.K. and H.W. Blanch. 1988. Enzymatic Production of L-Tryptophan in a Reverse Micelle Reactor. *Bioprocess Engineering* 3:83-91.

Eggers, D.K., D. J. Lim and H. W. Blanch. 1988. Enzymatic Production of L-Tryptophan in Liquid Membrane Systems. *Bioprocess Eng.* 3:23-30

Eggers, D.K., H.W. Blanch and J.M. Prausnitz. 1988. Extractive Catalysis: Solvent Effects on Equilibria of Enzymatic Reactions in Two-Phase Systems. *Enzyme and Microbial Technology* (in press).

England, C. 1984. Isothermal Separation Processes Update. JPL 9950-927, DOE/CS-66001-6 Prepared for Jet Propulsion Laboratory by Engineering Research West, Santa Monica, CA, August.

Garcia, A.A. and C.J. King. 1988. The Use of Polymer Sorbents for the Recovery of Acetic Acid from Dilute Aqueous Solution. LBL-24543, Lawrence Berkeley Laboratory, University of California, Berkeley, CA, January.

Goddard III, W.A., J.J. Low, B.D. Olafson, A. Redondo, Y. Zeiri, M.L. Steigerwald, E.A. Carter, J.N. Allison and R. Chang. 1984. The Role of Oxygen and Other Chemisorbed Species on Surface Processes for Metals and Semiconductors; Approaches to Dynamical Studies of Surface Processes. *Proceedings of the Symposium on the Chemistry and Physics of Electrocatalysis*, Vol. 84-12, pp. 63-95, The Electrochemical Society, Ind., Pennington, New Jersey.

Goddard III, W.A.. 1985. Theoretical Chemistry Comes Alive: Full Partner with Experiment. *Science* 227:917-923.

Guo, Y. J.M. Langlois, W.A. Goddard III. 1988. Electronic Structure and Valence-Bond Band Structure of Cuprate Superconducting Materials. *Science* 239:896-899.

Hatcher, H.J.. 1986. Biocatalysis Program Bioseparations Task. Summary Report, E.G. and G. Idaho, Inc., Idaho National Engineering Laboratory, Idaho Falls, ID, February.

Ingham, J.D.. 1983. Potential Membrane Applications to Biocatalyzed Processes: Assessment of Concentration Polarization and Membrane Fouling. JPL Publication 83-6.

Ingham, J.D.. 1984. Biocatalyst Processes for Production of Commodity Chemicals: Assessment of Future Research Advanced for N-Butanol Production. Jet Propulsion Laboratory, JPL Publication 84-60, July 1.

Ingham, J.D. and J.J. Eberhardt. 1988. Bioprocess Energy-Economics: Rates of Production and Concentration of Chemical Products. Jet Propulsion Laboratory, DOE, ECUT Program Bulletin, DOE/ECUT-88/1, PB88-900401, Jan-Feb, 1-11.

Kertes, A.S. and C.J. King. 1986. Extraction Chemistry of Fermentation Product Carboxylic Acids. Biotechnology and Bioengineering 28:269-282.

Khosla, C. and J.E. Bailey. 1988. Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant Escherichia coli. Nature 331:633-635.

Khosla, C. and J.E. Bailey. 1988. The Vitreoscilla Hemoglobin Gene: Molecular Cloning, Genetic Expression and Its Effect on in vivo Heme Metabolism in Escherichia coli. Molecular and General Genetics (in press).

King, C.J., et al.. 1987. Non-wetting Polymeric Adsorbents for Selective Uptake of Carboxylic Acids and Glycols. Presented at the Symposium, Fundamentals of Adsorption, Meeting of the American Chemical Society, Denver, Colorado, April 1987, and Foundation Conference on Advances in Separation Technology, West Germany, April.

King, C.J.. 1987. Chapter on Chemical Association in "Handbook of Separation Process Technology," R.W. Rousseau, ed., John Wiley and Sons.

Klibanov, A.M. 1986. Polyphenol Oxidase - Catalyzed Hydroxylation of Aromatic Compounds in Organic Solvents. Quarterly Report No. 1, Massachusetts Institute of Technology, Cambridge, MA., March.

Kuhn, R.H. and J.C. Linden. 1986. Effects of Temperature and Membrane Fatty Acid Composition on Butanol Tolerance of Clostridium acetobutylicum. Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May.

Lee, S.B. and J.E. Bailey. 1984. A Mathematical Model for Δv Plasmid Replication: Analysis of Copy Number Mutants. Plasmid 11:151.

Lee, S.B. and J.E. Bailey. 1984. A Mathematical Model for Δv Plasmid Replication: Analysis of Wild-Type Plasmid. Plasmid 11:166.

- Lee, S.B. and J.E. Bailey. 1984. Analysis of Growth Rate Effects on Productivity of Recombinant Escherichia coli Populations Using Molecular Mechanism Models. Biotechnol. Bioeng. 26:66.
- Lee, S.B. and J.E. Bailey. 1984. Genetically Structured Models for lac Promoter-Operator Function in the Chromosome and in Multicopy Plasmids: lac Operator Function. Biotechnology. Bioeng. 26:1372.
- Lee, S.B. and J.E. Bailey. 1984. Genetically Structured Models for lac Promoter-Operator Function in the Chromosome and in Multicopy Plasmids: lac Promoter Function. Biotechnol. Bioeng. 26:1383.
- Lee, S.B., A. Seressiotis and J.E. Bailey. 1985. A Kinetic Model for Product Formation in Unstable Recombinant Populations. Biotechnology and Bioengineering 27:1699-1709.
- Linden, J.C. and R.H. Kuhn. 1986. Biochemistry of Alcohol Effects on Clostridia. In Ethanol Toxicity at the Cellular Level, N. Van Uden, ed., CRC Press, Cleveland, OH,.
- Mariam, D.H., A.L. Williams and W.V. Dashek. 1988. Substrate specificity and synthesis or activation of Coriolus versicolor polyphenol oxidase. SIM News 38:44.
- Miskowski, V.M. "The Electronic Spectra of μ -peroxodicobalt (III) Complexes," Comments Inorg. Chem., (in press).
- Moore, N.L., A.L. Williams and W.V. Dashek. 1988. Partial purification of extracellular Coriolus versicolor polyphenol oxidase. SIM News 38:44.
- Naylor, A.M. and W.A. Goddard III. 1988. Simulations of Starburst Dendrimer Polymers. Polymer Preprints. 29:215-216.
- Naylor, A.M. and W.A. Goddard III. 1988. Application of Simulation and Theory to Biocatalysis and Biomimetics. In: Proceedings of the Symposium on Impact of Surface and Interfacial Structure on Enzyme Activity (American Chemical Society, Washington, DC).
- Peretti, S.W. and J.E. Bailey. 1987. Simulations of Host-Plasmid Interactions in Escherichia coli: Copy Number, Promoter Strength, and Ribosome Binding Site Strength Effects on Metabolic Activity and Plasmid Gene Expression. Biotechnol. Bioeng. 29:316.
- Peretti, S.W. and J.E. Bailey. 1986. A Mechanistically Detailed Model of Cellular Metabolism for Glucose-Limited Growth of Escherichia coli B/r-A. Biotechnol. Bioeng. 28:1672.

Peretti, S.W. and J.E. Bailey. 1988. Transcription from Plasmid Genes, Macromolecular Stability and Cell Specific Productivity in Escherichia coli Carrying Copy Number Mutant Plasmids. Biotechnol. Bioeng. (in press).

Peretti, S.W. and J.E. Bailey. 1988. Transient Response Simulations of Recombinant Microbial Populations. Biotechnol. Bioeng. (in press).

Ramelmeier, R.A. and H.W. Blanch. 1988. Peroxidase Catalyzed Oxidation of p-Anisidine with H₂O₂ in Toluene. Biotechnology and Bioengineering (in press).

Ramelmeier, R.A. and H.W. Blanch. 1988. Mass Transfer and Cholesterol Oxidase Kinetics in a Liquid-Liquid Two-Phase System", Biocatalysis (in press).

Reardon, K.F., T. Scheper, and J.E. Bailey. 1986. In situ Fluorescence Monitoring of Immobilized Clostridium acetobutylicum. Biotechnology Letters 8:817.

Riva, S., J. Chopineau, A.P.G. Kieboom and A.M. Klibanov. 1988. Protease-catalyzed Regioselective Esterification of Sugars and Related Compounds in Anhydrous Dimethylformide. J. Am. Chem. Soc. 110:584-589.

Rixey, W.G.. 1987. Ph.D. Dissertation. Non-wet Adsorbents for the Selective Recovery of Polar Organic Solvents from Dilute Aqueous Solution. University of California, Berkeley.

Scott, C.D.. 1987. Dispersed-Phase Adsorbents for Biotechnology Applications. Presented at 2nd Int'l Conference on Separation Tech., Germany, April 26-30.

Scott, C.D., B.H. Davidson, and J.E. Thompson. 1988. Use of Immobilized Microbial Membrane Fragments to Reduce Oxygen Content and Enhance Acetone-Butanol Fermentation. (in press).

Scott, C.D., C.A. Woodward, and J.E. Thompson. 1988. Solute Diffusion in Biocatalyst Gel Beads Containing Biocatalysts and Other Additives. Enzyme and Microbial Technology (in press).

Scott, C.D.. 1985. Techniques for Producing Mono-dispersed Biocatalyst Beads for Use in Columnar Bioreactors. In Proceedings of the Seventh Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TE, May 16.

Scott, C.D.. 1985. Innovative Techniques for Gel Immobilization of Microorganisms and Enzymes. International Conference on Enzyme Engineering, Helsingor, Denmark, September.

Scott, C.D. and J.E. Thompson. 1986. Mass Transfer Properties of Biocatalyst Beads. Eighth Symposium on Biotechnology for Fuels and Chemicals, Gatlingburg, Tennessee, May 13-16.

Seo, J.H. and J.E. Bailey. 1985. Effects of Recombinant Plasmid Content on Growth Properties and Cloned Gene Product Formation in Escherichia coli. Biotechnology and Bioengineering 27:1668-1674.

Seo, J.H., A. Loffler, and J.E. Bailey. 1988. A Parametric Study of Cloned Fusion Protein Expression in Escherichia coli. Biotechnol. Bioeng. (in press).

Seressiotis, A. and J.E. Bailey. 1987. Optimal Gene Expression and Amplification Strategies for Batch and Continuous Recombinant Cultures. Biotechnol. Bioeng. 29:392.

Seressiotis, A. and J.E. Bailey. 1986. MPS: An Algorithm and Data Base for Metabolic Pathway Synthesis. Biotechnol. Lett. 8:837.

Seressiotis, A. and J.E. Bailey. 1988. MPS: An Artificially Intelligent Software System for the Analysis of Metabolic Pathways. Biotechnology Bioengineering 31:587.

Shanks, J.V. and J.E. Baoley. 1988. Estimation of Intracellular Sugar Phosphate Concentrations in Saccharomyces cerevisiae Using ³¹P Nuclear Magnetic Resonance Spectroscopy. Biotechnology Bioengineering (in press).

Srienc, F., J.E. Bailey and J.L. Campbell. 1985. Effect of ARSI Mutations on Chromosome Stability in Saccharomyces cerevisiae. Mol. Cell. Biology 5:1676-1684.

Srienc, F., J.L. Campbell, and J.E. Bailey. 1986. Analysis of Unstable Recombinant Saccharomyces cerevisiae Population Growth in Selective Medium. Biotechnol. Bioeng. 28:996.

Srienc, F., J.L. Campbell, and J.E. Bailey. 1986. Flow Cytometry Analysis of Recombinant Saccharomyces cerevisiae Populations. Cytometry 7:132.

Srienc, F., J. Campbell and J.E. Bailey. 1984. Flow Cytometry Characterization of Plasmid Stability in Saccharomyces cerevisiae. Third European Congress on Biotechnology, Vol. III, P. 111-187, Verlag Chemie, Weinheim, Federal Republic of Germany.

Srienc, F., J.L. Campbell, J.E. Bailey. 1983. Detection of Bacterial β -Galactosidase Activity in Individual Saccharomyces cerevisiae Cells by Flow Cytometry. Biotechnology Letters 5:43.

Srienc, F., J.E. Bailey and J.L. Campbell. 1985. Effect of ARSI Mutations on Chromosome Stability in *Saccharomyces Cerevisiae*. *Mol. Cell. Biology* 5:1676-1684.

Steinmeyer, D., T. Cho, G. Efthymiou and M. L. Shuler. 1988. Extractive Fermentation in a Multimembrane Bioreactor. *Chem. Tech.* (in press).

System for Biotechnology Assessment. 1985. Final Report, Phase I: August 1984 - July 31, 1985, Washington University, St. Louis, MO., August.

Tamada, J.A., A.S. Kertes and C.J. King. 1986. Solvent Extraction of Succinic Acid from Aqueous Solutions. International Conference on Solvent Extraction, Munich, Germany, September.

Taylor, R., W.V. Dashek, A.L. Williams, G.C. Llewellyn, W.C. Shortle, and J.E. Mayfield. 1988. Ultrastructure of the Wood-Decay Fungus, Coriolus versicolor, in Relation to a Catechol-Induced Bimodal Growth Response. *International Biodeterioration* 24:(in press).

Taylor, R., G.C. Llewellyn, J.E. Mayfield, W.C. Shortle and W.V. Dashek. 1987. Attempts to determine whether the products of extracellular polyphenol oxidase modulate the catechol-induced bimodal growth response of Coriolus versicolor. *Biodeterioration Research I*, pp.43-62, Plenum Press, NY.

Taylor, R. G.C. Llewellyn, J.E. Mayfield, W.C. Shortle and W.V. Dashek. 1987. Time-dependent appearance of extracellular polyphenol oxidase in relation to the bimodal growth response of Coriolus versicolor to catechol. *Biodeterioration Research I*, pp.63-74, Plenum Press, NY.

Taylor, R., G.C. Llewellyn, C.E. O'Rear, J.E. J.E. Mayfield and W.V. Dashek. 1989. In vitro Growth of Coriolus Versicolor, A Wood-Decay Fungus, Responds Differentially to Catechol and Tannic Acid. Pam Am Section of International Biodeterioration Society, *Biodeterioration Research*. Plenum Press (in press).

The ECUT Biocatalysis Project: Jet Propulsion Laboratory, ECUT Program Bulletin, DOE, ECUT-86/1, PB86-900401, Jan-Feb, 1986.

The ECUT Biocatalysis Project: Jet Propulsion Laboratory, Submitted for Publication in the ECUT Program Bulletin, May, 1987.

Tomalia D.A., G.B. Kiefer, A.M. Naylor, W.A. Goddard III. 1988. Starburst Dendrimers V: Molecular Shape Control. *J. Amer. Chem. Soc.*, (in press).

Voecks, G.E.. 1983. Heterogeneous Catalysis Modelling Program Concept. Jet Propulsion Laboratory, DOE/CS-66001-2, August.

Warren, L.F.. 1984. Electrocatalysis Research - Final Report for the Period August 25, 1982 through December 30, 1983, JPL 9950-926, DOE/CS-66001-7, Prepared for Jet Propulsion Laboratory by Rockwell International, Science Center, Thousand Oaks, California, August.

Wilcox, R.E.. 1983. Industry, University, and Research Institute Interest in the U.S. Department of Energy ECUT Biocatalysis Research Activity. Jet Propulsion Laboratory, JPL Publication 83-90, DOE/CS-66001-3, November.

Williams, A.C., N.L. Moore, W.V. Dashek and A.L. Williams. 1988. Genetic engineering of Coriolus versicolor's polyphenol oxidase gene(s) and its industrial applications. USDA Symposium on Biotechnology, Alabama A & M University.

Wittrup, K.D. and J.E. Bailey. 1988. A Segregated Model of Recombinant Multicopy Plasmid Propagation. Biotechnol. Bioeng. 31:304.

Wittrup, K.D. and J.E. Bailey. 1988. A Mathematical Model of Recombinational Amplification of the dv Plasmid in the Yeast Saccharomyces cerevisiae. J. Theoret. Biol. 130:481.

Wittrup, K.D., M.B. Mann, D.M. Fenton, L.B. Tsai and J.E. Bailey. 1988. Single-Cell Light Scatter as a Probe of Refractile Body Formation in Recombinant Escherichia coli. Bio/Technology 6:423.

Wolfram, J.F.. 1985. Biologically Effected Separation Beneficiation. E.G. & G. Idaho, Inc., Idaho National Engineering Laboratory, Idaho Falls, ID, Quarterly Progress Report for January 1 to March 31.

Wolfram, J.F.. 1985. Bioseparation Project. E.G. & G. Idaho, Inc., Idaho National Engineering Laboratory, Idaho Falls, ID, Quarterly Status Report April 1 to June 30.

Wolfram, J.F.. 1985. Bioseparation Project. E.G. & G. Idaho, Inc., Idaho National Engineering Laboratory, Idaho Falls, ID, Quarterly Status Report July 1 to September 30.

Zaks, A. and A.M. Klibanov. 1988. Enzymatic Catalysis in Non-Aqueous Solvents. J. Biol. Chem. 263:3194-3201.

SECTION V

GUIDELINES FOR PROPOSALS

To assist potential investigators within the ECUT Biocatalysis Program, the following guidelines have been prepared. The first section involves statements regarding the mission and objectives of the program, the second gives proposal preparation guidelines and the third describes the evaluation process and criteria for proposal peer review.

Description of DOE-ECUT Biocatalysis Project Managed by JPL

The ECUT Biocatalysis Project supports the DOE Energy Conversion and Utilization Technologies Program goals by carrying out research to establish the technology base needed by the chemical processes industry to develop and implement genetically engineered processes for energy-efficient, competitive production of commodity chemicals from renewable feedstocks. Objectives include the investigation and development of new techniques and reaction sequences, with particular emphasis on solving problems in biocatalysis to improve energy productivity and reduce petroleum-based feedstock use in the production of chemicals.

The primary objective of the Biocatalysis Project is to resolve the critical technical constraints (e.g., poor productivity, high separation energetics, presence of aqueous medium, lack of design tools) that impede the utilization of biocatalysis for the production of chemicals and materials. To achieve this objective, the Biocatalysis Project supports generic applied research, as well as development of predictive theoretical models (that are experimentally verifiable) which address the technological barriers to the commercial utilization of biochemical catalysis.

The ECUT Biocatalysis Project consists of a few major work elements, each addressing a key technical component of the required enabling technology base. The selection, as well as the relationship between each of these work elements, has been defined by their scale of action.

The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysis and pursuing practical applications of basic molecular biology research findings.

The Process Design and Analysis work focuses on developing user-friendly computer programs which assess the energetics and economics of biocatalyst chemical production processes.

The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between cellular scale events and macro-level parameters.

Proposal Preparation Guidelines

All proposals may be submitted to either:

Dr. Leonard Keay,
Biocatalysis Program Manager
Energy Conversion and Utilization
Technologies (ECUT) Program
Conservation and Renewable Energy
Department of Energy CE-12
Forrestal Bldg. 5E-066
1000 Independence Avenue, S.W.
Washington, D.C. 20585
(202) 586-5377 (FTS) 896-5377

Dr. Minoo Dastoor
ECUT Biocatalysis Project
Project Manager
Jet Propulsion Laboratory
California Institute of Technology
M/S 125-112
4800 Oak Grove Drive
Pasadena, CA 91109
(818) 354-7429 (FTS) 792-7429

Proposals and preproposals may be submitted at any time during the year and will be reviewed as quickly as possible. There are no fixed formats for proposal preparation. It is suggested that the proposal be as short as possible to clearly define the proposed effort (suggested length of 15-20 pages excluding references, tables and figures, institutional requirements, and resumes). The following is given as a guide to your preparation.

- o Never assume that reviewers "will know what you mean."
- o Refer to relevant literature thoroughly and thoughtfully.
- o Explicitly state the rationale of the proposed investigation and its relevance to the ECUT Biocatalysis project mission and objectives.
- o Include well-designed tables and figures.
- o Present an organized, lucid write-up that clearly defines the objectives and desired accomplishments.
- o Submit three copies.

Review Process and Criteria

Upon submission of the proposal or preproposal, program managers at DOE will evaluate it for relevance to the ECUT Biocatalysis Project (see accompanying flow chart).

If the proposal is found to fit the project, it will be submitted for peer review by the ECUT Biocatalysis Project field office at Jet Propulsion Laboratory. Likewise, if a preproposal is found to be programmatically acceptable then a full proposal will be requested and the proposal will be subject to the peer review described above.

Based on peer review results, the proposal will either be rejected or approved for funding. If approved the proposal is evaluated programmatically for funding priority and assuming that funds are available, notification of acceptance will be given by JPL. The overall process from preproposal to acceptance will likely take 6-10 months. The actual awarding of funds will take an additional 8-12 weeks from the time of acceptance. Regardless of programmatic considerations, proposals with positive peer review results will be maintained on file for future consideration if resources become available (Figure 5-1).

The following are some of the major factors that will be considered in evaluating the proposal:

1. Relationship of the overall concept to the ECUT Biocatalysis project mission and goals.
2. Technical feasibility of the concept to actually address the ECUT Biocatalysis mission and goals.
3. Scientific and technical merit of the proposed effort.
4. Adequacy of approach and methodology to successfully carry out the proposed research.
5. Qualifications and experience of the principal investigator and staff.
6. Reasonableness of the proposed budget, duration and availability of resources.

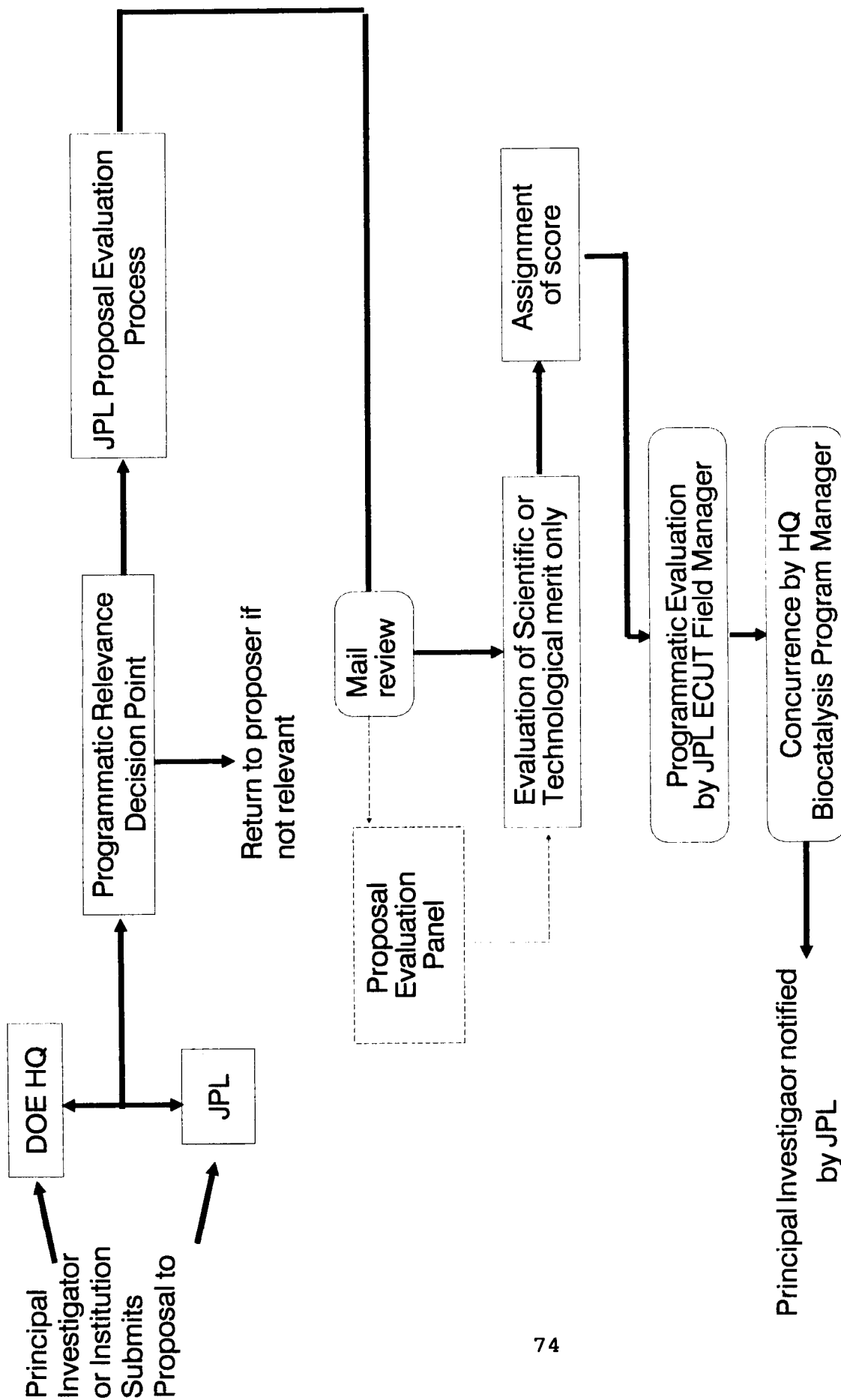


Figure 5-1. Proposal Evaluation Process

TECHNICAL REPORT STANDARD TITLE PAGE

1. Report No. 89-5		2. Government Accession No.		3. Recipient's Catalog No.	
4. Title and Subtitle ECUT Energy Conversion and Utilization Technologies Program: Biocatalysis Project Annual Report FY 1988				5. Report Date March 1989	
				6. Performing Organization Code	
7. Author(s) Larry Baresi				8. Performing Organization Report No.	
9. Performing Organization Name and Address JET PROPULSION LABORATORY California Institute of Technology 4800 Oak Grove Drive Pasadena, California 91109				10. Work Unit No.	
				11. Contract or Grant No. NAS7-918	
				13. Type of Report and Period Covered JPL External Publication	
12. Sponsoring Agency Name and Address NATIONAL AERONAUTICS AND SPACE ADMINISTRATION Washington, D.C. 20546				14. Sponsoring Agency Code	
15. Supplementary Notes					
<p>16. Abstract The Annual Report presents the fiscal year (FY) 1988 research activities and accomplishments, for the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Division. The ECUT Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology. The Biocatalysis Project is a mission-oriented, applied research and exploratory development activity directed toward resolution of the major generic technical barriers that impede the development of biologically catalyzed commercial chemical production. The approach toward achieving project objectives involves an integrated participation of Universities, Industrial Companies and Government Research Laboratories.</p> <p>The Project's technical activities were organized into three work elements:</p> <p>The Molecular Modeling and Applied Genetics work element includes research on modeling of biological systems; developing rigorous methods for the prediction of three-dimensional (tertiary) protein structure from the amino acid sequence (primary structure) for designing new biocatalysis; defining kinetic models of biocatalyst reactivity; and developing genetically engineered solutions to the generic technical barriers that preclude widespread application of biocatalysis.</p> <p>The Bioprocess Engineering work element supports efforts in novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yields and lower separation energetics. Results of work within this work element will be used to establish the technical feasibility of critical bioprocess monitoring and control subsystems.</p> <p>The Bioprocess Design and Assessment work element attempts to develop procedures for assessing the energy-economics of biocatalyzed chemical production processes.</p>					
17. Key Words (Selected by Author(s)) Biochemistry; Bioengineering; Chemical Engineering; Organic Chemistry; Energy (General); Conversion Techniques; Materials (General); Environmental Biology; Microbiology; Chemistry (General)				18. Distribution Statement Unclassified -- Unlimited	
19. Security Classif. (of this report) Unclassified		20. Security Classif. (of this page) Unclassified		21. No. of Pages 74	
				22. Price	